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(54) Title: AGRICULTURAL-CHEMICAL-PRODUCING ENDOSYMBIOTIC MICROORGANISMS AND METHOD OF PREPARING AND USING SAME

(57) Abstract

Hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with plants are disclosed. The microorganisms are prepared by combining genetic material from an agricultural-chemical-producing microorganism and a plant infecting microorganism to form hybrid microorganisms and selecting from the hybrid microorganisms those hybrid microorganisms which are capable of producing an agricultural chemical, which do ·not create manifestations of disease in the plant host, and which are capable of entering into an endosymbiotic relationship with the plant host. Methods of use are also disclosed.

AGRICULTURAL-CHEMICAL-PRODUCING ENDOSYMBIOTIC MICROORGANISMS AND METHOD OF PREPARING AND USING SAME

DESCRIPTION

Agricultural-Chemical-Producing Endosymbiotic
Microorganisms and Method of Preparing and Using Same

This application is a continuation-in-part of
application Serial No. 07/919,375, filed July 29, 1992, which
is a continuation of application Serial No. 07/466,465, filed
January 16, 1990, which is a continuation-in-part of
application Serial No. 07/266,232, filed October 28, 1988,
which is a continuation of application Serial No. 06/799,999,
filed November 20, 1985, now abandoned, which is a
continuation-in-part of application Serial No. 06/534,071,
filed September 20, 1983, now abandoned in favor of
continuation application Serial No. 07/166,819, filed
March 3, 1988, which itself is a continuation-in-part of
application Serial No. 06/484,560, filed April 13, 1983,
abandoned in favor of continuation application Serial No.
06/933,337, filed November 20, 1986, and now abandoned in
favor of continuation application Serial No. 07/266,221,
filed October 28, 1988.

The present invention relates to agricultural-chemical-
producing microorganisms, particularly agricultural-chemical-
producing bacteria capable of entering into endosymbiotic
relationships with host plants whereby the bacteria provide
some or all of the plant's agricultural-chemical
requirements.

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BACKGROUND OF THE INVENTION

Modern commercial agriculture is heavily dependent on the use of agricultural chemicals to enhance the performance of plants. Most notably, chemical fertilizers, particularly fixed-nitrogen fertilizers, are the most common limiting nutrient in crop productivity and often are the most expensive single input for the farmer. Other agricultural chemicals are widely used at great expense to kill plant pests, to prevent disease, or to improve the growing environment of the plant. Still other agricultural chemicals may be added to growing plants or harvested crops to diminish or enhance natural properties or to alter or improve the appearance or sensory appeal of the plant or crop. Agricultural chemicals include, as well, natural or synthetic plant growth regulators, including hormones and the like. Such chemicals are well known to those having ordinary skill in the agricultural art and are hereinafter generically referred to as "agricultural chemicals."

Microorganisms, such as bacteria, fungi, and algae are a natural source of agricultural chemicals. For example, leguminous plants, such as soybeans, alfalfa and clover, derive some of their fixed nitrogen requirements through symbiotic relationships with bacteria of the genus _Rhizobium_. Particular species of _Rhizobium_ infect the roots of leguminous plants forming nodules in which the bacteria are shielded from oxygen and provided with carbohydrate nutrients. In this anaerobic process, the rhizobia fix atmospheric nitrogen, which is then available for use by the plant.

Microorganisms have also become sources of agricultural chemicals synthesized or prepared by man and applied by spraying or the like onto fields, growing plants, or harvested crops. Antifungal antibiotics, antibacterial antibiotics, and insecticidal antibiotics, for example, have been produced by various bacteria. Antifungal antibiotics include blasticidin S which is produced by _Streptomyces griseochromogenes_, kasugamycin which is produced by

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Streptomyces kasugaensis, and polyoxins which are produced by
Streptomyces cacaoi var. asoensis. Antibacterial antibiotics
include streptomycin produced by Streptomyces griseus, and
tetracycline produced by Streptomyces viridifaciens.
Insecticidal antibiotics include tetranactin, produced by
Streptomyces aureus strain S-3466, and the bets-exotoxin and
delta-endotoxins produced by Bacillus thuringiensis. See
K. Aizawa, "Microbial Control of Insect Pests," and T. Misato
and K. Yoneyama, "Agricultural Antibiotics" in Advances in
Agricultural Microbiology, N.S.S. Rao, Editor (1982), which
is specifically incorporated herein by reference in its
entirety.

Similarly, microorganisms have become a source of
antibiotics for herbicide use. For example, cycloheximide,
produced by Streptomyces griseus, and herbicidin A and B,
produced by Streptomyces saganoensis, exhibit herbicidal
activity. See Y. Sekizawa and T. Takematsu, "How to Discover
New Antibiotics for Herbicidal Use" in Pesticide Chemistry,
J. Miyamoto and P.C. Kearney, Editors (1983), which is
specifically incorporated herein by reference in its
entirety.

Moreover, microorganisms are known to produce plant
growth regulating substances such as various vitamins,
auxins, cytokinins, gibberellin-like substances and other
stimulating or inhibiting substances. Brown, in her work on
"Seed and Root Bacterization" cited below, attributes the
production of such substances to species of Azotobacter,
Pseudomonas and Bacillus, including B. megaterium, and B.
subtilis.

Bacteria have been found that produce agents active
against a number of invertebrates, such as plant pathogenic
nematodes. One of these agents is Streptomyces avermitilis,
which produces avermectins. S. avermitilis and the
antiparasitic compounds it produces are described in U.S.
patents 4,310,519, 4,429,042, and 4,469,68, which are
specifically incorporated herein by reference in their
entirety.

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Microorganisms have also been found to produce antiviral antibiotics, including laurusin, which has been isolated from <u>Streptomyces lavendulae</u>, and miharamycin, which has been isolated from <u>Streptomyces miharuensis</u>. <u>See</u> T. Misato, K. Ko, and I. Yamaguchi, "Use of Antibiotics and In Agriculture" in <u>Advances in Applied Microbiology</u>, Vol. 21, (1977), which is specifically incorporated hereby by reference in its entirety.

Other types of bacteria are known to have the ability to solubilize phosphate that is otherwise unavailable to plants. These include those bacteria belonging to the genera <u>Bacillus</u> and <u>Pseudomonas</u>. Attempts to utilize this ability to these bacteria by inoculating seeds or plants with the bacteria have produced inconsistent results. <u>See</u> N.S.S. Rao, "Phosphate Solubilization by Soil Microorganisms" in <u>Advances in Agricultural Microbiology</u>, N.S.S. Rao, Editor (1982), which is specifically incorporated herein by reference in its entirety.

Few symbiotic associations between agricultural-chemical-producing microorganisms and major crop species, such as wheat and corn, have been described in the scientific literature. The development of symbiotic relationships between such microbial species and various crops would have many advantages over man made synthesis and application of the chemical. The advantages of such associations are particularly apparent in the areas of insecticides and fixed-nitrogen fertilizers, which are hereinafter discussed in detail as exemplary of the advantages attainable through the practice of the present invention. It should be understood however, that the teachings contained herein regarding bacterial production of insecticides and of fixed-nitrogen fertilizers may be extrapolated by those having ordinary skill in the art to other bacterially or microbially produced agricultural chemicals, such as those discussed above.

A particular advantage of the present invention is that the agricultural chemical is introduced within the entire plant or parts thereof rather than just on the surface as in

the case when certain agricultural chemicals are sprayed on
the plant. For example, insecticides that are sprayed on and
do not penetrate into the plant can be ineffective or of only
limited effectiveness against insects that bore into the
plant. However, the insecticide should be effective when
applied as a hybrid, endosymbiotic microorganism of the
present invention.

It is estimated that development of a symbiotic
relationship between nitrogen-fixing bacteria and major crop
species capable of providing even a minor fraction, e.g., 10-
20%, of the fixed nitrogen requirements of non-leguminous
crop plants, would produce an economically important
substitution of photosynthetically-derived energy for fossil
fuel energy now expended for production of nitrogen
fertilizer. Currently, the major sources of fixed nitrogen
for non-leguminous economic crop plants are man-mad products
such as anhydrous ammonia, inorganic nitrogen salts and
synthetic organic nitrogen compounds. Present annual
consumption of nitrogen fertilizer in the United States is
estimated to be 12 million tons. Such nitrogen fertilizers
are largely prepared by energy intensive processes for the
conversion of atmospheric nitrogen into ammonia.

A major thrust of nitrogen-fixation research in recent
years has been directed towards the isolation,
characterization, transformation and expression in plants of
the nitrogen-fixing genes from rhizobia and other nitrogen-
fixing bacteria by genetic modification of the plants
themselves. This technique requires a complete understanding
of the genetics and biochemistry of nitrogen fixation which,
it is estimated, is 10 to 15 years or more in the future.
See generally, J.E. Beringer, "Microbial Genetics and
Biological Nitrogen Fixation," in Advances In Agricultural
Microbiology, N.S.S. Rao, Editor (198), and G.P. Roberts
et al., "Genetics and Regulation of Nitrogen Fixation," Ann.
Rev. Microbiol., 35:207-35 (1981), both of which are
specifically incorporated herein by reference in their
entirety.

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Other types of bacteria besides <u>Rhizobium</u> are known to have the capability of fixing atmospheric nitrogen. These include those belonging to the genera <u>Azotobacter</u>, <u>Azomonas</u>, <u>Derxia</u> and <u>Beijerinckia</u>, which fix nitrogen aerobically, and those belonging to the genus <u>Klebsiella</u> which, like <u>Rhizobium</u>, fix nitrogen anaerobically. The literature is filled with reports of unsuccessful attempts to develop symbiotic relationships, similar to <u>Rhizobium</u>-legume relationships, between these bacteria and non-leguminous plants.

In the early 1940's, for example, it was proposed in the Soviet Union and elsewhere that nitrogen-fixing bacteria could be used as soil inoculants to provide some or all of the fixed nitrogen requirements of non-leguminous crop plants. Indeed, claims of increased crop yield associated with such inoculations were widespread. Critical analysis of the early yield results, however, revealed that positive significant effects occurred in only about one-third of the trials. It was later established that yield increases associated with bacterial inoculation, including inoculation with species of <u>Azotobacter</u>, were primarily due to changes in the rhizosphere microbial population, disease suppression by the inoculants, production of plant growth-promoting substances, and mineralization of soil phosphates. No ability to establish an associative symbiotic relationship with nitrogen-fixing bacteria in non-leguminous plants had been demonstrated. See M.E. Brown, "Seed and Root Bacterization", <u>Annual Review of Phytopathology,</u> <u>12</u>:181-197 (1974), which is specifically incorporated herein by reference in its entirety.

It has been demonstrated that an <u>artificial</u> symbiosis can be created between non-leguminous plant cells in tissue culture and aerobic nitrogen-fixing bacteria such as <u>Azotobacter vinelandii</u>. The demonstration involved forced association between higher plant and bacterial cells and did not provide a technique whereby a symbiotic association between such nitrogen-fixing bacteria and growing plants

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could be created. See P.S. Carlson, et al., "Forced
Association Between Higher Plant and Bacterial Cells In
Vitro," Nature, Volume 252, No. 5482, pp. 393-395 (1974),
which is specifically incorporated herein by reference in its
entirety.

Another effort to create higher organisms capable of
fixing their own nitrogen, for example, involved attempts to
create new organelles in higher organisms by similar forced
association of lower forms of organisms having nitrogen-
fixing abilities. Such efforts to parallel the theoretical
biological development of known organelles have included, for
example, the efforts of Burgoon and Bottino to induce the
uptake of nitrogen-fixing Gloecapsa sp. blue-green algae by
plant cells, the efforts of Davey and Cocking to induce the
uptake of nitrogen-fixing Rhizobium sp. bacteria into plant
cells, and the efforts of Giles to induce uptake of nitrogen-
fixing Azotobacter vinelandii bacteria by ectomycorrhizal
fungi. See A.C. Burgoon and P.J. Bottino, Journal of
Heredity, Volume 67, pp. 223-226 (1972); M.R. Davey and E.C.
Cocking, Nature, Volume 239, pp. 455-456 (1972); and
K.L. Giles, "The Transfer of Nitrogen-Fixing Ability to
Nonleguminous Plants," Plant Cell and Tissue Culture
Principles and Applications, W.R. Sharp et al., Editors
(1979). Neither the work of Burgoon and Bottino nor the work
of Davey and Cocking was successful in generating a nitrogen-
fixing higher organism. If the effects of Giles were
successful, which has not been conclusively established, they
would at best result in fungi with organelle-like structures
capable of fixing nitrogen which are capable only of living
on the outsides of roots of forest species rather than living
endosymbiotically with major crop plants.

Another attempt to create a symbiotic relationship
between nitrogen-fixing bacteria and non-leguminous plants
involved the screening and selection of plant hybrids in an
effort to identify some plant varieties which were capable of
associating with aerobic nitrogen-fixing bacteria, such as
Azotobacter vinelandii, in the soil. However, those efforts

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resulted in the identification of plant species which were
only about .5% as active as soybean plants inoculated with
rhizobia which were not deemed to be commercially useful.
See S.W. Ela, et al., "Screening and Selection of Maize to
Enhance Associative Bacterial Nitrogen Fixation," <u>Plant
Physiol.</u>, <u>70</u>:1564-1567 (1982), which is specifically
incorporated herein by reference in its entirety.

The numerous unsuccessful attempts to create associative
symbiotic relationships between nitrogen-fixing bacteria and
non-leguminous plants have resulted in the development of
numerous techniques for genetic manipulation and analysis.
These attempts have also resulted in rather extensive
characterization of the genetic makeup of nitrogen-fixing
bacteria such as <u>Azotobacter</u>. For example, it has been
discovered that mutant strains of <u>Azotobacter vinelandii</u>
lacking the nitrogen-fixation gene could be transformed into
bacteria having the capability to fix nitrogen by
introduction of genetic material from <u>Rhizobium</u> species. See
G.P. Roberts, "Genetics and Regulation of Nitrogen Fixation,"
<u>Ann. Rev. Microbiol.</u>, <u>35</u>:207-35 (1981). Moreover, new
techniques for protoplast fusion, which involve promotion of
interspecific genetic recombination by fusing two different
cells which have had their cell walls removed, were
recognized as providing a potentially attractive technique
for the production of new strains for commercial purposes
without requiring an understanding of the underlying
genetics. It was acknowledged in the scientific literature,
however, that it has yet to be determined whether or not
protoplast fusion can be used for genetic studies of gram-
negative nitrogen-fixing bacteria. See J.E. Beringer,
"Microbial Genetics and Biological Nitrogen Fixation,"
<u>Advances in Agricultural Microbiology</u>, N.S.S. Rao, Editor
(1982) at 13.

Indeed, experimentation related to protoplast fusion of
nitrogen-fixing bacteria has, prior to the present invention,
failed to produce evidence that non-pathogenic endosymbiotic
relationships between higher plants and fusion products of

nitrogen-fixing bacteria can be produced. For example, Du
Qian-you and Fan Cheng-ying, "A Preliminary Report on the
Establishment of Nitrogen Fixation System in the Crown Gall
of Tomato Plant," Acts Botanica Sinica, Vol. 23, No. 6
(1981), which is specifically incorporated herein by
reference in its entirety, described an attempt to effect
protoplast fusion between parent strains of Agrobacterium
tumefaciens (Pen^r, Ti) and Azotobacter chroococcum (Pen^s,
Nif$^+$). The hybrids were selected for nitrogen fixation and
their resistance to penicillin and were thereafter inoculated
on the stem of a tomato plant. The inoculation produced
crown galls or tumors found to possess nitrogenase activity
under aerobic conditions according to the acetylene reduction
test. The fusion products of the experiment produced a
pathogenic response in the host plant and were not shown to
be capable of entering into an endosymbiotic relationship
with the plant. Specifically, it could not be ascertained
whether the fixed nitrogen being produced in the vicinity of
the tumors was being used by the plant as a source of
nitrogen. Finally, it could not be determined whether the
nitrogenase activity observed was the result of a pathogenic
hybrid or of a penicillin resistant mutant of Azotobacter
remaining in a mixed inoculum. Moreover, it has now been
discovered that the attempts of Du Qian-you et al. to mimic
the nodule formation mechanism of the Rhizobium-legume
interaction by intentional inclusion of the tumor-forming Ti
plasmid in their hybrids were headed in the opposite
direction from that required to produce hybrid bacteria
capable of entering into successful endosymbiotic
relationships with plant hosts.

     Other unsuccessful attempts to extend the range of
nitrogen-fixing Rhizobium species include the insertion of
the Ti plasmid from Agrobacterium tumefaciens into selected
Rhizobium strains. See P.J.J. Hooykass, et al., J. Gen.
Microbiol., 98:477-484 (1977), which is specifically
incorporated herein by reference in its entirety. These
studies resulted in Rhizobium strains able to produce galls

on non-leguminous dicotyledonous plants which will fix
nitrogen but only under anaerobic conditions not naturally
occurring in such plants. The criticisms outlined in the
previous paragraph also applies to this work.

Despite substantial economic incentive to do so, the
prior art has been unable to prepare hybrid nitrogen-fixing
bacteria capable of entering into endosymbiotic relationships
with non-leguminous plant hosts or any other hybrid
agricultural-chemical-producing microorganisms capable of
entering into endosymbiotic relationships with plant hosts.

SUMMARY OF THE INVENTION

A method of producing hybrid agricultural-chemical-
producing microorganisms capable of entering into
endosymbiotic relationships with a plant host has been
discovered which comprises combining genetic material of an
agricultural-chemical-producing microorganism and a plant-
infecting microorganism which infects the plant host
(hereinafter sometimes referred to as an "infecting
microorganism") to form hybrid microorganisms and selecting
from the hybrid microorganisms those hybrid microorganisms
which are capable of producing an agricultural chemical and
of entering into an endosymbiotic relationship with the plant
host and which do not create manifestations of disease in the
plant host.

The hybrid microorganisms are formed by combining some
or all of the genetic material of the agricultural-chemical-
producing microorganism and the infecting microorganism. The
genetic material is combined through use of various
techniques, such as recombinant DNA, recombinant RNA, cell
fusion, conjugation, plasmid transfer, transformation,
transfection, transduction, and microinjection.

The microorganisms resulting from the process of the
present invention may be introduced into crop plants by a
variety of means, including injection, and may be employed to
coat agricultural seed, to infect agricultural seed, in the
preparation of a soil drench, and the like. The hybrid
microorganisms of the present invention, upon association

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with crop plants, are capable of supplying some or all of the plants' agricultural chemical needs. Agricultural chemicals that can be produced by hybrid bacteria in accordance with the present invention include fertilizers, particularly fixed-nitrogen fertilizers; antibiotics, including antibacterial, antiviral, antifungal, insecticidal, nematocidal, miticidal, and herbicidal agents; plant growth regulators, including plant hormones, and the like.

In a preferred embodiment, there has been discovered a method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprising:

    (A)   in any convenient order;

        (1)   identifying a plant host;

        (2)   identifying an infecting microorganism which infects the plant host;

        (3)   selecting mutants of the infecting microorganism with one or more selectable traits in addition to ability to infect the host; and

        (4)   selecting an agricultural-chemical-producing microorganism having one or more selectable traits;

    (B)   forming fusion hybrids of the infecting microorganism and the agricultural-chemical-producing microorganism;

    (C)   selecting serially from the products of the previously performed step or substep and in any convenient order:

        (1)   a subgroup comprising those hybrids having both at least one of the selectable traits of the agricultural-chemical-producing microorganism and at least one of the selectable traits of the infecting microorganisms;

        (2)   a subgroup comprising those hybrids which manifest the ability to interact with plant

tissue in the manner in which the infecting
microorganism interacts with plant tissue
during the initial phase of infection in the
plant host;

(3)   a subgroup comprising those hybrids which,
upon application to the host, do not create
manifestations of a disease; and

(4)   a subgroup comprising those hybrids having the
ability to produce the agricultural chemical
if not previously selected for; and

(D) selecting hybrid agricultural-chemical-producing
microorganisms capable of entering into
endosymbiotic relationships with a plant host by
selecting from the products of the last performed
step of stages (C)(1) to (C)(4) those hybrid
microorganisms capable of improving the performance
of the plant host under conditions wherein its
performance could be improved by direct application
of the agricultural chemical or the agricultural-
chemical-producing microorganism to the plant.

In a particularly preferred embodiment, the hybrid
microorganisms are hybrid bacteria produced by protoplast or
spheroplast fusion of an infecting bacterium and an
agricultural-chemical-producing bacterium having the same
response to gram stain. Certain gram-positive bacteria,
notably members of the genus _Bacillus_, _Streptomyces_, and
Clavibacter are particularly useful for the formation of the
preferred hybrid bacteria.

In another preferred embodiment, the hybrid
agricultural-chemical-producing microorganisms of the present
invention capable of entering into endosymbiotic
relationships with a plant host are formed by the steps
comprising:

(A) in any convenient order:

(1)   identifying the plant host; and

(2)   identifying an infecting microorganism which
infects the plant host;

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(B)   preparing a vector capable of being transferred into and replicating in the infecting microorganism;

(C)   preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;

(D)   placing the expression module in the vector to create an expression vector capable of being transferred into and replicating in the infecting microorganism, the expression vector being capable of directing the production of the agricultural chemical by the infecting microorganism;

(E)   transforming the infecting microorganism with the expression vector to produce hybrid microorganisms;

(F)   selecting for the hybrid microorganisms;

(G)   selecting serially from the selected hybrid microorganisms and in any convenient order:

    (1)   a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;

    (2)   a subgroup comprising those hybrid microorganisms which, upon application to the host, do not create manifestations of disease; and

    (3)   a subgroup comprising those hybrid microorganisms having the ability to produce the agricultural chemical if not previously selected for; and

(H)   selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance

of the plant host under conditions wherein the
performance would be improved by direct application
of the agricultural chemical or the agricultural-
chemical-producing microorganism to the plant host.

In a particularly preferred embodiment, the hybrid
agricultural-chemical-producing microorganisms capable of
entering into endosymbiotic relationships with a plant host
are formed by the steps comprising:

    (A)   in any convenient order:

        (1)   identifying a plant host; and

        (2)   identifying an infecting microorganism
            which infects the plant host;

    (B)   preparing an integration vector containing an
        integration sequence and capable of
        integrating into the genome of the infecting
        microorganism;

    (C)   preparing an expression module capable of
        directing the production of an agricultural
        chemical by the infecting microorganism;

    (D)   placing the expression module within the
        integration sequence of the integration
        vector, thereby producing a modified
        integration vector capable of integrating into
        the genome of the infecting microorganism and
        directing the production of the agricultural
        chemical by the infecting microorganism;

    (E)   transforming the infecting microorganism with
        the modified integration vector to produce
        hybrid microorganisms;

    (F)   selecting for the hybrid microorganisms;

    (G)   selecting serially from the selected hybrid
        microorganisms and in any convenient order:

        (1)   a subgroup comprising those hybrid
            microorganisms which manifest the ability
            to interact with plant tissue in the
            manner in which the infecting
            microorganism interacts with plant tissue

during the initial phase of infection in
the plant host;

(2)   a subgroup comprising those hybrid
microorganisms which, upon application to
the host, do not create manifestations of
disease; and

(3)   a subgroup comprising those hybrid
microorganisms having the ability to
produce the agricultural chemical, if not
previously selected for; and

(H)   selecting hybrid agricultural-chemical-
producing microorganisms capable of entering
into endosymbiotic relationships with a plant
host by selecting from the products of the
last performed step of steps (G)(1) to (G)(3)
those hybrid microorganisms capable of
improving the performance of the plant host
under conditions wherein the performance would
be improved by direct application of the
agricultural chemical or the agricultural-
chemical-producing microorganism to the plant
host.

The processes are capable of producing agricultural-
chemical-producing microorganisms capable of entering into
endosymbiotic relationships with both monocotyledonous and
dicotyledonous plants. For dicotyledonous plants, infecting
bacteria of the genus Agrobacterium are preferred, with
strains of Agrobacterium tumefaciens being particularly
preferred. Species of the genus Erwinia, such as Erwinia
carotovora may also be used, as may species of the genus
Pseudomonas, such as Pseudomonas solanacearum and Pseudomonas
syringae, of the genus Xanthomonas, such as Xanthomonas
campestris, and of the genus Streptomyces, such as S.
ipomoea. For monocotyledonous plants, species of the genus
Erwinia, such as Erwinia stewartii, are preferred infecting
bacteria. Species of the genus Xanthomonas, such as
Xanthomonas campestris, species of the genus Azospirillum,

such as <u>Azospirillum lipoferum</u> and <u>Azospirillum brasilense</u>,
and species of the genus <u>Pseudomonas</u>, such as <u>Pseudomonas
syringae</u>, are also contemplated as being useful. <u>Pseudomonas
syringae</u> is contemplated as being particularly useful as an
infecting bacterium for the formation of fusion products
applicable to cereals, including temperate cereals and rice.
<u>Clavibacter</u> species such as <u>C. xyli</u> subsp. <u>xyli</u> and <u>C. xyli</u>
subsp. <u>cynodontis</u> are particularly useful for grasses, such
as maize, sorghum, and the like.

Preferred agricultural-chemical-producing microorganisms
and the chemicals and/or applications for which their
metabolic products are useful are identified in Table I below
and include organisms having the ability to produce
fertilizers, including fixed nitrogen and chemicals capable
of solubilizing phosphates, antibiotics, including
antibacterial compounds, antifungal compounds, antiviral
compounds, insecticides, nematocides, miticides and
herbicides, and plant growth regulators. Additionally,
useful organisms may be selected or modified to produce other
agricultural chemicals, as above defined, including
fragrances, antifeeding agents and the like.

The processes of the present invention result in novel,
stable hybrid microorganisms having the ability to produce
one or more agricultural chemicals and enter into
endosymbiotic relationships with a plant host. The nitrogen-
fixing hybrid bacteria of the present invention, for example,
have been shown to improve the yield of non-leguminous crop
plants growing under low fixed nitrogen conditions by amounts
of from 10 to 180%, due primarily, it is believed, to fixed
nitrogen produced by the hybrids of nitrogen-fixing bacteria
and infecting bacteria in the course of their endosymbiotic
relationship with the plant host.

Additional objects and advantages of the invention will
be set forth in part in the description which follows, and in
part will be obvious from the description, or may be learned
from the practice of the invention. The objects and
advantages may be realized and attained by means of the

instrumentalities and combinations particularly pointed out
in the appended claims.

The hybrid agricultural-chemical-producing
microorganisms produced by the above-described method may be
further modified by natural or artificial genetic techniques
to improve their performance as sources of agricultural-
chemical for the plant host. Such modification could result,
for example, in the ability to excrete the agricultural
chemical, such as fixed nitrogen, including the ability to
excrete the agricultural chemical in a particular form, such
as fixed nitrogen in the form of amino acids; the ability to
continue production of the agricultural chemical even in the
presence of adequate amounts of that chemical from other
sources; in a reduction of the hybrid's resistance to cold
temperature (i.e., to prevent unintended proliferation of the
hybrids from year to year); in enhancement of the hybrid's
ability to withstand drought, disease, or other physiological
stress; in the introduction of additional agricultural-
chemical-producing functions; or in modification of the
hybrid so that it cannot grow outside the plant host.

While the steps of the above-described method may be
carried out in any convenient order, it is desirable that the
process of selection for agricultural-chemical-producing
ability and for ability to interact with plant tissue in the
manner in which the infecting microorganism interacts with
plant tissue during the initial phases of infection in the
host plant be carried out two or more times before the step
relating to selection of those hybrids which do not manifest
symptoms of disease in the plant host.

The selectable traits associated with the infecting
bacterium may be antibiotic resistance, need for specific
nutritional supplementation (auxotrophism), resistance to
toxins, or the like. The selectable traits associated with
the agricultural-chemical-producing microorganism may be the
ability to produce an agricultural chemical alone or in
combination with one or more of those traits previously
mentioned with respect to the infecting microorganism. The

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interaction with plant tissue screened for in the above-described method may be, for example, the ability to bind to plant tissue, the ability to spread throughout the vascular system of the plant, or the like. Where the interaction associated with the initial phase of infection is binding to plant cells, it is desirable to limit further the microbial subgroup resulting from the penultimate step by selection of a microbial subgroup for further screening in accordance with the above-described process comprising those hybrids which spread most readily throughout the plant host.

By appropriate selection of the infecting microorganism, hybrids may be obtained which are capable of entering into endosymbiotic relationships with cereals, such as wheat, triticale, barley, rye, rice and oats; grasses, such as brome grass, blue grass, tall fescue grass, fine fescue grass, ryegrass, and Bermuda grass; tropical grasses, such as sugar cane, corn, millet and sorghum; solanaceous plants, such as potatoes, tomatoes, tobacco, eggplant and pepper; brassicaceous plants such as cauliflower, broccoli, cabbage, kale and kohlrabi; other vegetables, such as carrot and parsley; other agriculturally grown plants, such as sugar beets, cotton, fruit trees, berry plants, and grapes; and economically important tree species, such as pine, spruce, fir and aspen. The process of the present invention and the resulting microorganisms may also be used to fulfill some or all of the fixed-nitrogen or other agricultural chemical requirements of leguminous plants, such as soybeans, alfalfa, clover, field beans, mung beans, peas and other pulses, as a supplement, for example, to fixed nitrogen provided by species of <u>Rhizobium</u> associated with nodules on their roots.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one embodiment of the invention and, together with the description, serve to explain the principles of the invention.

Figure 1 depicts a partial restriction map of the plasmid pCG300.

Figure 2 depicts a partial restriction map of the plasmid pCG306, which is derived from pCG300.

Figure 3 depicts a partial restriction map of the plasmid pCG6.

Figure 4 depicts a truncated <u>B. thuringiensis</u> delta endotoxin gene fused to a kanamycin resistance gene in the vector m8TK65.

Figure 5 depicts the insertion of a truncated <u>B. thuringiensis</u> delta endotoxin gene fused to a kanamycin resistance gene into pCG6 Neo^S and the insertion of an expression module containing these genes into pCG300.

Figure 6 show an abbreviated restriction map of the delta-endotoxin gene of <u>B. thuringeinsis</u> subsp. <u>kurstaki</u> HD73 and flanking sequences.

Figure 7 shows the sequence of 4.993 HincII Fragment of Tn916.

Figure 8 shows the restriction map of Tn916.

Figure 9 shows a Tn916 restriction map with insertions of Tn5 and the resulting effects on behavior.

Figure 10 shows the construction of pCG563.

Figure 11 shows the modification of the delta-endotoxin gene of <u>B. thuringeinsis</u> subsp. <u>kurstaki</u> HD73.

Figure 12 shows the altered and native sequences of the delta-endotoxin gene of <u>B. thuringeinsis</u> subsp. <u>kurstaki</u> HD73 and flanking sequences.

Figure 13 shows the construction of the Bt integration plasmid pCG741.

Figure 14 shows the restriction maps of four probes, the Bt probe, the TetM probe, the pGEM probe, and the Cxc 209 probe.

Figure 15 shows the restriction enzyme sites used for southern analysis of pCG741 in MDR1.586.

Figure 16 shows the results of a Southern hybridization analysis of DNA.

Figure 17 shows the results of a Southern hybridization analysis of DNA samples.

Figure 18 shows the population dynamics of a Cxc/Bt construction in greenhouse corn.

Figure 19 shows a standard curve generated by scanning densitometry of immunovisualized Western blot.

Figure 20 shows a densitometry of Western blots.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Reference will now be made in detail to the presently preferred embodiments of the invention, which together with the following examples, serve to explain the principles of the invention.

As noted above, the present invention relates to a method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host which comprises combining genetic material from an agricultural-chemical-producing microorganism and an infecting microorganism which infects the plant host to form hybrid microorganisms and selecting from the hybrid microorganisms those capable of producing the agricultural chemical, which do not create manifestations of a disease in the plant host, yet which are capable of entering into an endosymbictic relationship with the plant host. Preferably, the hybrid agricultural-chemical-producing microorganisms are capable of improving the performance of the plant host under conditions wherein the performance would be improved by direct application of the agricultural chemical or the agricultural chemical-producing microorganism to the plant. Most preferably, the agricultural-chemical-producing microorganism and the infecting microorganism are bacteria. As used throughout this specification, the term "direct application" means application to the whole plant or any part of the plant, including systemic or partial systemic application.

The term "microorganism" as used herein is intended to encompass bacteria, fungi (including yeast), and algae. The term "bacteria" as used herein is intended to encompass

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bacteria, bacteria-like organisms and their equivalents,
including gram positive bacteria, gram negative bacteria,
actinomycetes and the like. The only limitations on the
taxonomic classification of the microorganisms used in
accordance with the present invention is that some or all of
their genetic material be capable of being used to produce
viable hybrid organisms expressing phenotypical properties of
both parents as more fully described herein.

"Infecting microorganisms" as used throughout this
specification is intended to connote not only microorganisms
which enter and live within the plant and normally produce
symptoms of disease but also microorganisms which enter and
live within the plant symbiotically or commensally. Indeed,
some of the plant infecting microorganisms used in accordance
with the present invention, while normally creating
pathogenic responses in some plant hosts, will not normally
produce such responses in all plant hosts.

Performance can be determined and evaluated by those
skilled in the art by considering any one or more of a
multitude of factors. These include: 1) resistance to
environmental stress, such as drought, high salinity, pests,
and harmful chemicals; 2) increased yield; 3) faster
maturity; 4) less dependence on added nutrients; and 5)
enhanced quality of the plant product of interest.

The hybrid microorganisms are formed by combining some
or all of the genetic material of the agricultural-chemical-
producing microorganism and the infecting microorganism. The
genetic material is combined by the techniques of recombinant
DNA, recombinant RNA, cell fusion, conjugation and plasmid
transfer, transformation, transfection, transduction, and
microinjection. Some of these techniques are described in
Maniatis, T., E.F. Fritsch, and J. Sambrook, Molecular
Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory
1982) and Miller, J.H., Experiments in Molecular Genetics
(Cold Spring Harbor Laboratory 1972), both of which are
specifically incorporated herein by reference in their
entirety. Selection of a technique by which the hybrid

microorganisms of the present invention may be formed will
generally be within the capabilities of one of ordinary skill
in the art based on the above-referenced scientific
literature and the teachings disclosed herein.

In a preferred embodiment, the agricultural-chemical-
producing microorganisms of the present invention capable of
entering into endosymbiotic relationships with a plant host
may be formed by steps comprising:

(A)   in any convenient order:

(1)   identifying the plant host;

(2)   identifying an infecting microorganism which
infects the plant host;

(3)   selecting mutants of the infecting
microorganism with one or more selectable
traits in addition to ability to infect the
host; and

(4)   selecting an agricultural-chemical-producing
microorganism having one or more selectable
traits;

(B)   forming fusion hybrids of the infecting
microorganism and the agricultural-chemical-
producing microorganism;

(C)   selecting serially from products of the previously
performed step or substep and in any convenient
order:

(1)   a subgroup comprising those hybrids having
both at least one of the selectable traits of
the agricultural-chemical-producing
microorganism and at least one of the
selectable traits of the infecting
microorganism;

(2)   a subgroup comprising those hybrids which
manifest the ability to interact with plant
tissue in the manner in which the infecting
microorganism interacts with plant tissue
during the initial phase of infection in the
plant host;

(3)   a subgroup comprising those hybrids which,
        upon application to the host, do not create
        manifestations of a disease; and

(4)   a subgroup comprising those hybrids having the
        ability to produce the agricultural chemical
        if not previously selected for; and

(D) selecting hybrid agricultural-chemical-producing
     microorganisms capable of entering into
     endosymbiotic relationships with a plant host by
     selecting from the products of the last performed
     step of steps (C)(1) to (C)(4) those hybrid
     microorganisms capable of improving the performance
     of the plant host under conditions wherein the
     performance could be improved by direct application
     of the agricultural chemical or the agricultural-
     chemical-producing microorganism to the plant.

     As used in the steps above, the term "selecting" means
any intervention or combination of interventions such that
the desired microorganism can be recognized either by its
ability to survive or by its unique properties.

     As indicated, the steps of the method may be performed
in any convenient order. Preferably, they are performed in
the order recited with the selectable traits of the
agricultural-chemical-producing microorganism screened for in
step (C)(1) including the ability of the hybrid to produce
the agricultural chemical in question, as in the case of
fixed-nitrogen producing bacteria. If screening for ability
to produce the agricultural chemical in question is not
conducted in the first screening step, then it is preferred
to conduct such screening (step C(4)) prior to or
concurrently with the screening in the plant host
contemplated by steps (C)(2) and (C)(3). When performed in
the order recited, the antibiotic resistance markers or the
like constituting the selectable traits associated with the
infecting bacterium need not survive subsequent screening
procedures.

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The stable hybrid microorganisms resulting from processes of the present invention are distinguished from hitherto known organisms in that they have both the ability to produce agricultural chemicals and the ability to enter into endosymbiotic relationships with a plant host. The endosymbiotic relationship referred to is one in which the organism actually exists within and spreads throughout all or a portion of the plant host, without causing a pathogenic response, deriving some or all of its energy requirements from carbohydrates and other materials produced by the plant host and providing agricultural chemicals which may be used by the plant host to supplement those otherwise available.

The plant hosts with which the microorganisms of the present invention may establish endosymbiotic relationships may include virtually all economically important crop plants. In addition, any particular hybrid microorganism is not necessarily limited to one particular species of plant host. Its host range will depend upon many factors, including the infecting microorganism from which it is derived. At the same time, the phenotypic traits controlled by the genetic material of the infecting microorganism from which it is derived will place limits on its host range, as will other traits specifically engineered into the hybrid organism.

The process of the present invention has been shown to be capable of producing stable hybrid microorganisms having the ability to produce an agricultural chemical and of entering into endosymbiotic relationships with both monocotyledonous and dicotyledonous plant hosts. A particularly important group of plant hosts for which the products of the present invention may serve as endosymbiotic agricultural-chemical-producing microorganisms are the cereals, including temperate cereals, such as wheat, triticale, barley, rye, rice and oats. Endosymbiotic agricultural-chemical-producing microorganisms formed in accordance with the present invention may also be useful in supplementing the agricultural chemical, including fixed nitrogen, needs of economically important sod and forage

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grasses, such as brome grass, blue grass, tall fescue grass and Bermuda grass. Similarly, the organisms of the present invention have a demonstrated ability to increase the yields of tropical grasses, such as sugar cane, corn, millet and sorghum. Solanaceous plants, such as potatoes, tomatoes, tobacco, eggplant and pepper are suitable plant hosts to which the organisms of the present invention may be applied, as are brassicaceous plants, such as cauliflower, broccoli, cabbage, kale and kohlrabi. Miscellaneous vegetables such as carrots and parsley; other agriculturally grown plants, such as sugar beets, cotton, fruit trees, berry plants and grapes; and economically important tree species, such as pine, spruce, fir and aspen, may also serve as plant hosts for the organisms of the present invention.

Even though plants may have symbiotic relationships with microorganisms which produce agricultural chemicals, such as leguminous plants with bacteria of the genus Rhizobium which fix atmospheric nitrogen anaerobically in root nodules, these plants are obtained in the greatest yield when provided with supplemental agricultural chemical sources through fertilization or otherwise. Accordingly, it is envisioned that such supplemental agricultural chemicals, including supplemental fixed nitrogen, for such plants may be provided by microorganisms formed in accordance with the present invention capable of producing agricultural chemicals, including fixed nitrogen, and capable of entering into endosymbiotic relationships with these leguminous plants.

The agricultural-chemical-producing microorganism employed in the present invention may be any microorganism that produces an agricultural chemical or chemical effect of interest. Agricultural-chemical-producing microorganisms that may be employed in the present invention are those capable of producing antibiotics, antifungal agents, antiviral agents, insecticides, nematocides, miticides, herbicides, plant growth regulating compounds, fertilizing chemicals other than fixed nitrogen, fragrances, sensory enhancing chemicals, antifeeding agents and the like.

Selection of such agricultural-chemical-producing
microorganism will be within the capabilities of those
skilled in the art in light of the teachings and
illustrations contained herein.

In one embodiment, the agricultural-chemical-producing
microorganism is a bacterium capable of fixing atmospheric
nitrogen aerobically. Such a bacterium may be selected from
the genera Azotobacter, Azomonas, Beijerinckia, and Derxia,
among others. A preferred group of nitrogen fixing bacteria
are those from the genus Azotobacter, such as Azotobacter·
vinelandii, Azotobacter paspali, Azotobacter beijerinckia and
Azotobacter chroococuum. Azotobacter vinelandii is
particularly preferred because its genetics and regulation of
nitrogen fixation have been extensively studied and because
it has a demonstrated ability to accept and express genetic
material from other bacterial genera. See G.P. Roberts, et
al., "Genetics and Regulation of Nitrog.n Fixation," Ann.
Rev. Microbiol., 35:207-35 (1981). Although Azotobacter and
other significant aerobic nitrogen-fixing bacteria are
generally gram-negative bacteria, it is to be
understood that the techniques of the present invention are
applicable to both gram-positive and gram-negative bacteria.

A table of exemplary agricultural-chemical-producing
bacteria contemplated as useful in accordance with the
present invention and the agricultural chemical or chemical
effects they produce appear in Table I.

Table I. Agricultural-Chemical-Producing Microorganisms

Group	Genus	Species	Chemical Application
Antibiotics Anti- fungal Agents	Streptomyces	cacaoi var. asoensis	produces polyoxins which prevent rice sheath blight
		griseus	produces cycloheximide which prevents

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		onion downy mildew
	griseo-chromagenes	produces blasticidin S which prevents rice blast
	hygrosco-picus var. limoneus	produces val-idamycin A which prevents rice sheath blight
	kasugaensis	produces kasugamycin which prevents rice blast
	kitazawaensis	produces ezomycin which prevents stem rot of kidney beans
	Tendae Tu/901	produces nik-komycin which is active against various fungi
Pseudomonas	_fluorescens strain B10_	causes iron to become unavailable to other micro-organisms such as fungi
Streptover-ticillium	_rimofaciens_	produces mildiomycin which is active against various

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Antibac- terial Agents	Streptomyces	chibaensis	powdery mildews produces cel- locidin which prevents rice bacterial leaf blight
		griseus	produces streptomycin which prevents various bacterial diseases
		lavendulae No. 6600 Gc-1	produces laurus in which prevents bacterial leaf blight of rice plants
		spheroides	produces novobiocin which prevents tomato canker
		venezuelae	produces chloramphenicol which presents bacterial leaf blight of rice plants
		viridi- faciens	produces tetracycline which prevents various bacterial diseases

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	Agrobacterium	radio-bacteria strain 84	produces agrocin - 84
	Nocardia	sp.	produces myomycin
Antiviral Agents	Streptomyces	hygrosco-picus var. aabomyce-ticus	produces aabomycin A which inhibits TMV multiplication in tobacco tissue
		lavendulae No. 6600 GC-1	produces laurusin which inhibits TMV multiplication in tobacco tissue
		miharuensis	produces miharamycin which is effective in controlling TMV, CMV, PVX, and RSV
Insecti-cides	Bacillus	cereus	affects hymenoptera lepidoptera, coleoptera
		euloomarahae	effects coleopteran larvae, especially Japanese beetle

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	fribou-gensis	affects coleopteran larvae, especially Japanese beetle
	lentimorbus	affects coleopteran larvae, especially Japanese beetle
	moritai	affects seed corn maggot
	popilliae	affects coleopteran larvae, especially Japanese beetle
	thurin-giensis var. alesti, dendro-limus, entomo-cidus, kurstaki, sotto, tenebrionis, israelensis, berliner	affect numer-ous insect pest larvae
Clostridium	bravi-daciens	affects tent caterpillar
	malacusomae	affects tent caterpillar

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	Streptomyces	strain B-41-146	produces milbemycin
		avermitilis	produces avermactins
Nematocide	Bacillus	penetrans	effective against root-knot nematodes
	Streptomyces	avermitilis	produces avermectins
Miticide	Steptomyces	aureus S-3466	produces tetranactin
		avermitilis	produces avermectins
Herbicides	Streptomyces	griseus	produces cycloheximide
		saganoensis	produces herbicidin A, B
		sp.	produces bialaphos
		sp.	produces anisymycin
	Pseudomonas	syringae pv. atropurpurae	produces cor-onatine which causes choco-late spot disease on Italian ryegrass
Fertilizers Nitrogen-fixing	Azotobacter	beijerin-ckia, enroo-coccum, paspali, vinelandii	fix atmospheric nitrogen
	Azomonas		fix atmospheric nitrogen
	Beijerinckia		fix atmospheric nitrogen

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	Derxia		fix atmospheric nitrogen
Phosphate Solubilizing	Bacillus	circulans, floure- scens, magaterium, mesen- tericus, mycoides, polymyxa, pulvi- taciens, subtilis	solubilize bound phos- phate
	Pseudomonas	calcis, liqui- faciens, putida, rathonia, striata	solubilize bound phos- phates
Plant Growth Reg- ulators	Azotobacter	sp.	produce vari- ous vitamins auxins, cyto- kinins, giberellin-like substance and other plant hormones
	Agrobacterium	sp.	
	Pseudomonas	sp.	
	Bacillus	megaterium, subtilis	

The infecting microorganism capable of infecting the plant host employed in the present invention may be any of a wide variety of bacterial species which infect the plant host under consideration. Preferably, the infecting microorganism should have a known method of interaction with the plant host during the initial phase of infection. The infecting

microorganism may be either a pathogen, including latent
pathogens, or an endosymbiotic species. In the case of
pathogens, it is preferred that the pathogen create a visible
manifestation of the disease associated with it. Exemplary
pathogens include species of the genera <u>Agrobacterium</u> and
<u>Erwinia</u>. Exemplary endosymbiotic species include species of
<u>Azospirillum</u>, <u>Corynebacterium</u>, and <u>Clavibacter</u>. The species
of <u>Azospirillum</u> are known to live in the roots of tropical
grasses, such as sugar cane, and temperate grasses, such as
wheat, without causing manifestations of disease. Certain
species of <u>Corynebacterium</u> live in wheat and corn. It has
been discovered that a species of <u>Clavibacter</u> lives in corn.

    Most preferably, the infecting microorganism is a strain
which is specific, or nearly specific, to the particular crop
plant host under consideration in order to limit the
potential for spread of the hybrid microorganisms of the
present invention to plants other than those with which they
are intended to enter an endosymbiotic relationship. Many
plant infecting microorganisms and their methods of
interaction with plant hosts are described in the scientific
literature. Particular reference in this regard is made to
M.S. Mount et al., <u>Phytopathogenic Prokaryotes</u>, Volumes I and
II (1982), which is specifically incorporated herein by
reference. In addition, xylem-limited bacteria, such as the
bacterium that causes Pierce's disease and that which
inhabits C-4 grasses such as Bermuda grass, will be useful in
the practice of this invention. Such bacteria are described
in Wells, et al., <u>Phytopathology</u> 71:1156-1161 (1981) and
Raju, et al., <u>Am. J. Enol. Vitic.</u> 31:17-21 (1980), which are
incorporated herein by reference.

    Many pathogenic microorganisms are known to form strains
essentially undifferentiable from each other except for their
preference for particular plant hosts. Such host-specific
pathogen strains are known as pathovars for the plant host
involved. It is envisioned that the present invention will
be particularly useful with pathovars for the particular host
under consideration, including pathovars of the genera

Agrobacterium, particularly Agrobacterium tumefaciens;
Erwinia, particularly Erwinia stewartii and Erwinia
carotovora; Pseudomonas, particularly Pseudomonas
solanacearum and Pseudomonas syringae; Xanthomonas,
particularly Xanthomonas campestris; and similar bacteria.

Particularly preferred non-pathogenic endosymbionts
include species of the genus Azospirillum, particularly
Azospirillum lipoferum and Azospirillum brasilense, the genus
Acremonium, particularly Acremonium typhinum and Acremonium
coenophialum, and the genus Balansia. Infecting
microorganisms envisioned as suitable for use in accordance
with the present invention include those listed in Table II.

Table II.   Plant-Infecting Microorganisms

Type	Genus	Species
Gram-negative bacteria aerobic rods and cocci	Pseudomonas	agarici, andropogonis, avenae, caricapapayae, caryophylli, cichorii, cissicola, gladioli, glumae, marginalis, pseudoalcaligenes subsp. citrulli, rubrilineans, rubrisubalbicans, solanacearum, syringae, tolaasii, viridiflava
	Xanthomonas	albilineans, ampelina, axonopodis, campestris, fragariae
	Agrobacterium	rhizogenes, rubi, tumefaciens
Facultatively anaerobic rods	Erwinia	amylovora, ananas, cancerogena, carnegieana, carotovora subsp. atroseptica, carotovora subsp.

		carotovora, chrysanthemi, cypripedii, dissolvens, herbicola, mallotivora, milletiae, nigrifluens, nimipressuralis, quercina, rhapontici, rubrifaciens, salicis, stewartii, tracheiphila, uredovora
Gram-negative bacteria	Azospirillum	lipoferum, brasilense
Gram-positive bacteria Actinomycetes and related organisms	Coryne-bacterium	betae, beticola, fascians, flaccumfaciens, ilicis, insidiosum, michiganense, nebraskense, oortii, poinsettiae, sepedonicum
	Clavibacter	xyli
	Streptomyces	ipomoea, scabies
Fungi	Acremonium	typhinum, coenophialum

Pathovars of Agrobacterium tumefaciens are particularly preferred for formation of stable hybrids capable of producing agricultural chemicals and of entering into endosymbiotic relationships with dicotyledonous plant hosts. Agrobacterium tumefaciens has a well identified interaction with the plant during the initial phases of infection in that it binds to plant cell tissue. The ability of Agrobacterium tumefaciens to bind to plant cell tissue in vitro has been

demonstrated and has been shown to parallel the host range
for tumor formation by Agrobacterium tumefaciens in vivo.
See A.G. Matthysse, et al., "Plant Cell Range for Attachment
of Agrobacterium tumefaciens to Tissue Culture Cells,"
Physiological Plant Pathology, 21:381-387 (1982); A.G.
Matthysse, et al., "Binding of Agrobacterium tumefaciens to
Carrot Protoplasts," Physiological Plant Pathology, 20:27-33
(1982), both of which are specifically incorporated herein by
reference.

Agrobacterium tumefaciens is the preferred infecting
microorganism for use in forming stable hybrids capable of
producing agricultural chemicals and of entering into
endosymbiotic relationships with solanaceous plants, such as
potatoes, tomatoes, tobacco, eggplant and pepper;
brassicaceous plants, such as cauliflower, broccoli, cabbage,
kale and kohlrabi; vegetables, such as carrot and parsley and
other agriculturally grown plants, such as sugar beets,
cotton, fruit trees, berry plants and grapes. Other
infecting microorganisms suitable for use in accordance with
the present invention to produce agricultural-chemical-
producing microorganisms capable of entering into
endosymbiotic relationships with dicotyledonous plants
include Erwinia carotovora, Pseudomonas solanacearum,
Pseudomonas syringae and Xanthomonas campestris.

Infecting microorganisms such as Pseudomonas syringae
and Xanthomonas campestris are envisioned as being
particularly useful in the formation of stable hybrid
microorganisms capable of producing agricultural chemicals
and of entering into endosymbiotic relationships with
monocotyledonous cereal crops, such as wheat, barley, rye,
rice and oats, and also with grasses, such as brome grass,
blue grass, tall fescue grass and Bermuda grass.

Corynebacterium and Clavibacter species are useful as
the infecting microorganism for such monocots as grasses,
wheat, corn, and sorghum.

Strains of Erwinia stewartii and Clavibacter xyli subsp.
cynodontis or subsp. xyli are particularly preferred as the

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infecting microorganism useful in forming stable hybrid
microorganisms capable of producing agricultural chemicals
and of entering into endosymbiotic relationships with
monocotyledonous plants, such as tropical grasses, such as
sugar cane, corn, millet and sorghum, and small grains such
as rice. Strains of <u>Pseudomonas syringae</u> and <u>Xanthomonas
campestris</u> are also envisioned as useful in these
applications.

The above-identified strains of <u>Azospirillum</u> are
envisioned as being useful in monocotyledonous plants,
including tropical and temperate grasses, such as sugar cane
and wheat, among others.

Species of the genus <u>Balansia</u> and the genus <u>Acremonium</u>,
particularly <u>Acremonium typhinum</u> and <u>Acremonium coenophialum</u>,
are useful as the infecting microorganism for grasses and
forages, such as tall fescue, fine fescue, and ryegrass.

<u>Streptomyces</u> species are useful as the infecting
microorganism of root and tuber crops, such as sweet potato,
white potato, sugar and other beets, and radishes.

It is to be understood that the selection of a
particular plant-infecting microorganism and a particular
agricultural-chemical-producing microorganism for use in
accordance with the present invention will be within the
capabilities of one having ordinary skill in the art through
the exercise of routine experimentation in light of the
teachings contained herein and inferences logically to be
drawn from the disclosure of the present invention. The
characteristics of microorganisms contemplated as useful as
either the agricultural-chemical-producing microorganism or
the infecting microorganism in processes of the present
invention may be determined by routine experimentation or by
reference to standard compendia, such as <u>Bergey's Manual of
Determinative Bacteriology</u>, and the like.

For use in the present invention, it is preferred that
mutants of the desired infecting microorganism be selected
which have one or more selectable traits in addition to the
ability to infect the plant host under consideration.

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Specifically, mutants of the infecting microorganism should
be selected which have traits which are selectable in vitro.
Such traits include antibiotic resistance, the need for
specific nutritional supplementation (auxotrophism),
resistance to toxins such as heavy metals, combinations
thereof, and the like, which will allow rapid in vitro
screening of the hybrid microorganisms to identify those
containing genetic material from the mutant infecting
microorganism. In the absence of a selectable trait,
physical means can be used to kill one or both parents but
which will allow hybrids to survive. Such physical means
include ultraviolet light and heat.

    Antibiotic resistance is the preferred selectable trait,
and it is particularly preferred that the selected infecting
mutant microorganism have resistance to at least two
antibiotics. Dual antibiotic resistance, or redundancy in
other selectable traits, ensure that the initial screening of
the hybrids will identify only those having genetic material
from both the agricultural-chemical-producing microorganism
and the selected infecting mutant microorganism and reduces
the chances of the survival or formation of mutant antibiotic
resistant strains of the agricultural-chemical-producing
microorganism. This is of particular value with fusion
hybrids and is of little or no significance if cloned genes
are employed. For example, in formation of agricultural-
chemical-producing hybrids capable of producing fixed
nitrogen and of entering into endosymbiotic relationships
with monocotyledonous plants, strains of Erwinia stewartii
which have resistance to both streptomycin and tetracycline
are preferred. In the formation of hybrids capable of
producing fixed nitrogen and of entering into endosymbiotic
relationships with dicotyledonous plants, strains of
Agrobacterium tumefaciens which are both streptomycin
resistant and kana/mycin resistant are preferred. Such
mutant strains of plant infecting microorganisms are
available from microorganism depositories such as the
American Type Culture Collection in Rockville, Maryland, or

may be generated from the wild type by techniques well known
in the art.

Where hybrids of the agricultural-chemical-producing
microorganism are not to be screened initially for the
ability to produce the agricultural chemical in question, the
agricultural-chemical-producing microorganism may be a mutant
with one or more additional selectable traits, preferably in
vitro selectable traits, such as those mentioned above with
respect to the infecting microorganism. Thus, for example,
if the agricultural chemicals of interest is an insecticide,
nematocide or the like, the agricultural-chemical-producing
microorganism may be a mutant which has selectable antibiotic
resistance or sensitivity or a selectable need for specific
nutritional supplementation. Obviously, these traits should
not be identical to the selectable traits of the infecting
microorganism since the initial screen would otherwise be
unable to eliminate non-hybrid organisms. While the presence
in the hybrids of such additional selectable traits from the
agricultural-chemical-producing microorganism does not
guarantee the presence of agricultural-chemical-producing
capability in the products of the initial screening
procedure, it sufficiently enriches the population with true
hybrid organisms to the point where there is a reasonable
predictability of success in identifying those hybrid
organisms with the agricultural-chemical-producing capability
in subsequent screening.

In a particularly preferred embodiment, the hybrids of
the present invention are formed by protoplast or spheroplast
fusion of bacteria following the outline of techniques
generally employed in the prior art. Known protoplast and
spheroplast fusion techniques are described in D.A. Hopwood,
"Genetic Studies With Bacterial Protoplasts," Ann. Rev.
Microbiol, 35:237-72 (1981), and in R.L. Weiss, J.
Bacteriol., 128:668-70 (1976), both of which are specifically
incorporated herein by reference in their entirety. In this
embodiment, it is preferred, although not necessary, that the

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infecting bacterium and the agricultural-chemical-producing
bacterium employed exhibit the same response to gram stain.

In general, the fusion procedure involves the removal of
the cell wall from both the agricultural-chemical-producing
bacteria and the infecting bacteria, fusion of the infecting
bacteria and agricultural-chemical-producing bacteria cells
in a fusion-inducing medium, such as polyethylene glycol, and
regeneration of the cell wall about the fusion hybrids
containing genetic material from both the infecting bacterium
and the agricultural-chemical-producing bacterium. Fusion
and regeneration of the cell wall are conducted at low
temperatures so that rates of expressible genetic
recombination are favored in relation to rates of enzymatic
destruction of genetic material in the newly formed hybrids.

Following initial formation of fusion hybrids, the
genetic make-up of the bacterial population is relatively
unstable for a period of two or three days, during which much
genetic recombination apparently occurs. During this time
period, those stable fusion hybrid capable both of
manifesting the one or more selectable traits associated with
the agricultural-chemical-producing bacteria and of
manifesting the one or more selectable traits associated with
the infecting bacterium are selected. It is particularly
preferred that the agricultural-chemical-producing trait also
be selected during this step. For example, fusion products
of Azotobacter vinelandii and Erwinia stewartii formed from
strains of Erwinia stewartii which are streptomycin and
tetracycline resistant are grown in a nitrogen-free medium
containing both streptomycin and tetracycline. Thus, only
those organisms containing both the genetic material
associated with nitrogen fixation from Azotobacter vinelandii
and the genetic material associated with streptomycin and
tetracycline resistance from Erwinia stewartii will survive
the two to three day incubation period.

The surviving fusion hybrids manifesting both the
selectable traits associated with the agricultural-chemical-
producing bacterium and the selectable traits associated with

the infecting bacterium may then be screened for their
ability to interact with plant tissue in the manner in which
the infecting bacterium interacts with plant tissue during
the initial phase of infection of the plant host. As noted
above, the initial phase of infection associated with
Agrobacterium tumefaciens is binding to plant tissue cells.
The ability of the hybrids to bind to plant tissue cells may
be determined in vitro by techniques hitherto described in
the above-cited literature and by techniques described in the
following examples.

Other injecting bacteria, such as Erwinia stewartii,
initially interact with plant tissue following infection by
spreading throughout the vascular system of the plant without
being detected and destroyed by the plant's disease-response
system. This character is assumed in the literature to be
due to an extracellular polysaccharide produced by the
infecting bacterium which permits it to elude the plant's
normal defensive reaction. The ability of the fusion hybrids
to spread throughout the vascular system of the plant in this
manner may be determined in any convenient manner. One
preferred screening technique involves the infection of
seedlings of the host plant at a particular site in the
plant. After a period of time, e.g., four days, the plant
may then be dissected into a plurality of transverse sections
displaced along the longitudinal axis of the plant.
Bacterial cultures may be regenerated from the bacteria
contained in each section. The bacteria-containing sections
farthest removed from the situs of initial infection will
contain those fusion hybrids best able to disperse throughout
the vascular system of the plant, thereby allowing selection
of hybrids having this ability.

It is to be understood that other techniques for
screening for this initial interaction with plant tissue may
be envisioned by those skilled in the art in light of the
teachings contained herein. It is to be further understood
that other mechanisms of initial interaction between
particular infecting bacteria and particular plant hosts may

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be quantified and screened for in ways which will be obvious
to those skilled in the art in light of the teachings
contained herein and known interactions between infecting
bacteria and their plant hosts.

The ability to spread throughout the plant's vascular
system is a desirable property even for fusion hybrids formed
from infecting bacteria whose initial interaction with plant
cell tissue is by some other mechanism, e.g., cell binding.
Accordingly, it is preferred to screen further those fusion
hybrids found to possess both the ability to produce
agricultural chemical and the ability to interact with plant
cell tissue in the manner in which the infecting bacterium
interacts with the plant host during the initial phases of
infection to select a subgroup of hybrids for further
screening according to the present invention which are also
capable of dispersing quickly throughout the vascular system
of the plant.

Moreover, in practicing this embodiment of the present
invention, it is preferred that the fusion hybrids found to
have both the ability to produce agricultural chemicals and
the ability to interact with plant cell tissue in the manner
in which the infecting bacterium interacts with plant cell
tissue during the initial phases of infection be screened for
these capabilities two or more times. Such repeated
screening tends to assure stability of the fusion hybrid
strain and also allows selection for further screening of a
manageable number of fusion hybrids having the greatest
capabilities in these two areas.

The stable fusion hybrids manifesting both the
selectable traits of agricultural-chemical-producing
bacterium and plant-tissue-interaction capabilities, as above
described, may then be grown as individual colonies, in the
case of the agricultural-chemical-producing bacterium that
fix nitrogen, preferably on nitrogen-free media. Each of the
resultant cultures may then be applied in an appropriate
manner, e.g., by injection, to seedlings of the plant host in
question. After an appropriate incubation period, e.g., two

or three weeks, those fusion hybrids which do not create any visible manifestation of a disease in the host plant seedlings may be selected for further screening. Experience has shown that screening of 25 to 30 of the best fusion hybrids is normally sufficient to provide 5 to 7 fusion hybrids which do not manifest visible disease symptoms, such as any associated with the original infecting bacterium, in the host plant in question.

Preferably, those stable fusion hybrids which manifest the widest area of spread throughout the vascular system of the plant, the clearest freedom from symptoms of disease in the host plant, and, in the case of agricultural-chemical-producing bacteria that fix nitrogen, the most vigorous growth in nitrogen-free media, are selected for further screening.

Some of the fusion products of the present invention have also been observed to form spores. Selection of endosymbiotic bacteria formed in accordance with the present invention which form spores facilitates application and survival of the bacteria when applied to plant hosts since the spores are generally more resistant to stress than the growing bacterial cells.

In another preferred embodiment, the agricultural-chemical-producing microorganisms of the present invention capable of entering into endosymbiotic relationships with a plant host are formed by the steps comprising:

    (A)   in any convenient order:
        (1)   identifying the plant host; and
        (2)   identifying an infecting microorganism which infects the plant host;
    (B)   preparing a vector capable of being transferred into and replicating in the infecting microorganism;
    (C)   preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;

(D)   placing the expression module in the vector to
        create an expression vector capable of being
        transferred into and replicating in the
        infecting microorganism, the expression vector
        being capable of directing the production of the
        agricultural chemical in the infecting
        microorganism;

(E)   transforming the infecting microorganism with
        the expression vector to produce hybrid
        microorganisms;

(F)   selecting for the hybrid microorganisms;

(G)   selecting serially from the selected hybrid
        microorganisms and in any convenient order:

    (1) a subgroup comprising those hybrid
        microorganisms which manifest the ability to
        interact with plant tissue in the manner in
        which the infecting microorganism interacts with
        plant tissue during the initial phase of
        infection in the plant host;

    (2) a subgroup comprising those hybrid
        microorganisms which, upon application to the
        host, do not create manifestations of disease;
        and

    (3) a subgroup comprising those hybrid
        microorganisms having the ability to produce the
        agricultural chemical if not previously selected
        for; and

(H)   selecting hybrid agricultural-chemical-producing
        microorganisms capable of entering into
        endosymbiotic relationships with a plant host by
        selecting from the products of the last
        performed step of steps (G)(1) to (G)(3) those
        hybrid microorganisms capable of improving the
        performance of the plant host under conditions
        wherein the performance would be improved by
        direct application of the agricultural chemical

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or the agricultural-chemical-producing
microorganism to the plant host.

As used in the steps above, the term "selecting" means
any intervention or combination of interventions such that
the desired microorganism can be recognized either by its
ability to survive or by its unique properties.

A vector capable of being transferred into and
replicating in an infecting microorganism may be prepared by
one skilled in the art in view of the teachings of the
present invention. As used herein, the term "prepared"
includes obtaining existing vectors known to have the desired
properties.

An expression module is prepared using techniques known
in the art. As used herein, an "expression module" is a DNA
sequence capable of directing the production of a product by
a cell, in this case an agricultural chemical by the
infecting microorganism. It comprises a portable DNA
sequence containing the structural gene or genes for the
production of an agricultural chemical and transcription and
translation control elements, the control elements being
operable in the infecting microorganism. The expression
module may also contain a DNA sequence that codes for a
selectable trait in the infecting microorganism. That
sequence may be controlled by the transcription and
translation control elements previously mentioned or it may
have its own transcription and translation control elements
that are operable in the infecting microorganism. It is
preferred that the selectable trait be antibiotic resistance.

As used herein, the term "portable DNA sequence" is
intended to refer either to a synthetically produced
nucleotide sequence or to a restriction fragment of a
naturally occurring DNA sequence. This sequence is obtained
from the previously discussed agricultural-chemical-producing
microorganisms. The methods of obtaining such sequences will
be apparent to those of ordinary skill in the art in view of
the teachings of the present invention and methods known in
the art. Examples of such methods may be found in Maniatis,

T., et. al, <u>Molecular Cloning, A Laboratory Manual</u> (Cold
Spring Harbor Laboratory 198), which is specifically
incorporated herein by reference in its entirety.

One preferred way of making an expression module is as
follows. A portable DNA sequence containing the structural
gene or genes for the agricultural chemical is prepared. The
portable sequence is then cloned into a vector containing
transcription and translation control elements for the
portable DNA sequence. These control elements are operable
in the infecting microorganism. If the portable DNA sequence
and the control elements are properly aligned by techniques
known to those skilled in the art, an expression module is
created in this vector. The expression module is capable of
directing the infecting microorganism to produce the
agricultural chemical. The module is then recovered by
techniques known in the art.

There are alternative ways of making an expression
module. For example, some or all of the control elements may
be attached to the portable DNA sequence prior to its being
cloned into the vector, in which case the vector need not
contain those elements.

The transcription and translation control elements, as
discussed herein, include at least one promoter, at least one
ribosome binding site, at least one translation initiation
codon, and at least one translation termination codon. These
elements may also include stability enhancing sequences and
any other sequence necessary or preferred for appropriate
transcription and subsequent translation of the vector DNA.
These elements can include synthetic DNA, portions of natural
DNA sequences, or products of <u>in vitro</u> mutagenesis. The
particular control elements that are chosen for incorporation
are chosen by criteria that are frequently empirically
determined. That is, different configurations of control
elements, for example, the ribosome binding site and its
flanking sequences, are tested for their effect upon
expression directly until a successful configuration is

evident. Such empirical determination involves techniques known to those skilled in the art.

After the expression module has been prepared, it is placed in the vector that is capable of being transferred into and replicating in the infecting microorganism, creating an expression vector. The expression vector is capable of being transferred into and replicating in the infecting microorganism and capable of directing the production of the agricultural chemical by that microorganism.

This vector is transferred into the infecting microorganisms to produce hybrid microorganisms by techniques known to those of ordinary skill in the art in view of the teachings of the present invention. Not all of the infecting microorganisms will be transformed by the vector. Therefore, it will be necessary to select for the hybrid microorganisms. Such selection techniques will be apparent to those of ordinary skill in the art in light of the teachings of the present invention. One of the preferred selection techniques involves incorporating into the expression vector a DNA sequence, with any necessary transcription and translation control elements, that codes for a selectable trait in the infecting microorganism, if such a sequence is not already in the expression module. A preferred selectable trait is antibiotic resistance.

Once the hybrid microorganisms have been selected, it is necessary to select for those that are hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host. This is done by following the steps of (G)(1)-(G)(3) and (H) above as described herein.

In a particularly preferred embodiment, the agricultural-chemical-producing DNA sequence is cloned into an integration vector, which is capable of integrating into the genome of the infecting microorganism. Use of such an integration vector prevents the transmission of the cloned DNA sequence to other microorganisms by plasmid transmission.

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In particular, this method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprises:

    (A)   in any convenient order:

        (1)   identifying a plant host; and

        (2)   identifying an infecting microorganism which infects the plant host;

    (B)   preparing an integration vector containing an integration sequence and capable of integrating into the genome of the infecting microorganism;

    (C)   preparing an expression module capable of directing the production of an agricultural chemical by the infecting microorganism;

    (D)   placing the expression module within the integration sequence of the integration vector, thereby producing a modified integration vector capable of integrating into the genome of the infecting microorganism and directing the production of the agricultural chemical by the infecting microorganism;

    (E)   transforming the infecting microorganism with the modified integration vector to produce hybrid microorganisms;

    (F)   selecting for the hybrid microorganisms;

    (G)   selecting serially from the selected hybrid microorganisms and in any convenient order:

        (1)   a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which the infecting microorganism interacts with plant tissue during the initial phase of infection in the plant host;

        (2)   a subgroup comprising those hybrid microorganisms which, upon application to

                    the plant host, do not create
                    manifestations of disease; and

         (3) a subgroup comprising those hybrid
              microorganisms having the ability to
              produce the agricultural chemical, if not
              previously selected for; and

    (H) selecting hybrid agricultural-chemical-producing
         microorganisms capable of entering into
         endosymbiotic relationships with a plant host by
         selecting from the products of the last
         performed step of steps (G)(1) to (G)(3) those
         hybrid microorganisms capable of improving the
         performance of the plant host under conditions
         wherein the performance would be improved by
         direct application of the agricultural chemical
         or the agricultural-chemical-producing
         microorganism to the plant host.

As used in the steps above, the term "selecting" means
any intervention or combination of interventions such that
the desired microorganism can be recognized either by its
ability to survive or by its unique properties.

The integration vector can be prepared by techniques
known in the art. It must have a DNA sequence homologous to
a natural DNA sequence from the organism whose genome is the
integration target. This sequence is known as the
integration sequence. In a particularly preferred
embodiment, the integration vector is pCG300, which has been
deposited in the American Culture Collection, 12301 Parklawn
Drive, Rockville, MD 20852, U.S.A. and assigned Accession
No. 53329.

The expression module may be prepared as previously
described herein or by other techniques known in the art. It
should be noted that a selectable trait need not be in the
expression module. It may be elsewhere in the integration
vector, but it is preferably within the integration sequence.

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For certain embodiments of the present invention, it is
preferred that the expression module be prepared by:

1) preparing a vector containing a promoter that is
   operable in the infecting microorganism;
2) placing into the vector a portable DNA sequence
   containing the structural gene or genes for the
   production of the agricultural chemical and
   containing transcription and translation control
   elements other than the promoter, which are operable
   in the infecting microorganism, to produce an
   expression vector containing an expression module,
   wherein the expression module is operable in the
   infecting microorganism; and
3) recovering the expression module from the expression
   vector.

As previously mentioned, the control elements and the
portable DNA sequence will need to be properly positioned by
techniques known in the art in order to create an operable
expression module.

The vector prepared by the first step in the preceding
paragraph is often referred to as a promoter vector. Such a
promoter vector is useful for moving a promoter from the
infecting microorganism for the creation of the expression
module. A particularly preferred promoter vector is the
plasmid pCG6, which has been deposited in the American Type
Culture Collection, 12301 Parklawn Drive, Rockville,
MD 20852, U.S.A. and given the Accession No. 53328.

As previously mentioned, there are alternative ways to
make expression modules useful in the practice of the present
invention that will be apparent to those skilled in the art
in view of the teachings herein. In addition, the
transcription and translation control elements may be
obtained in several ways. They may be associated with the
structural gene or genes in the portable DNA sequence, or
they may also be created synthetically or by site-specific
mutagenesis.

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When the desired expression module is produced, it is
removed and cloned into the integration vector, preferably
within the integration sequence. The objective is that
through double cross-over events flanking the expression
module, only that portion of the integration vector
comprising the expression module will be integrated into the
genome of the infecting microorganism. In the event that the
expression module has been cloned outside of the integration
sequence, the entire integration vector may be integrated.
In either event, the modified integration vector is capable
of directing the production of the agricultural chemical by
the infecting microorganism.

The modified integration vector is then used to
transform the infecting microorganism to produce hybrid
microorganisms. Since only some of the infecting
microorganisms will be transformed, it is necessary to select
for such hybrid microorganisms. There are many such
selection techniques known to those of ordinary skill in the
art. A preferred selection technique is to select for a
marker or a trait such as antibiotic resistance in the hybrid
microorganism.

Once they have been selected, the subgroups of hybrid
microorganisms specified in steps (G)(1)-(G)(3) are selected
for serially as described herein. Then, hybrid agricultural-
chemical-producing microorganisms capable of entering into
endosymbiotic relationships for the plant host are selected
by selecting those hybrid microorganisms capable of improving
the performance of the plant host under conditions wherein
the performance would be improved by direct application of
agricultural chemical or the agricultural-chemical-producing
microorganism to the plant as described herein.

For the embodiments of the present invention involving
the use of recombinant DNA techniques, the plant hosts with
which the hybrid microorganisms may establish endosymbiotic
relationships, the infecting microorganisms, and the
microorganisms that are the source of the agricultural
chemical producing gene or gene include all of those

mentioned previously herein. However, it is preferred that
the infecting microorganism and the microbial source of the
agricultural chemical producing gene or genes are bacteria.
Most preferably, the infecting bacterium is a species of the
genus Corynebacterium or of the genus Clavibacter.
Clavibacter xyli subsp. cynodontis and Clavibacter xyli
subsp. xyli are especially preferred. When the infecting
bacterium is a species of the genus Clavibacter, it is
preferred that the plant host be of the Gramineae family. In
this case, particularly preferred hosts include Bermuda
grass, sugar cane, sorghum, and corn.

A preferred agricultural chemical is the delta endotoxin
of Bacillus thuringiensis. Various Bacillus thuringiensis
delta-endotoxin genes have been described, for example, in
Klier, A., et al., The EMBO Journal, 7:791-799 (1982) and
Schnepf, H.E., et al., Proc. Natl. Acad. Sci. USA, 5: 2893-
2897 (1981), both of which are specifically incorporated
herein by reference in their entirety. A preferred source of
the gene for the delta toxin is the M13 vector mBTK65,
deposited in the American Type Culture Collection, 12301
Parklawn Drive, Rockville, Maryland 20852, U.S.A. under
Accession No. 53326. It contains a truncated B.
thuringiensis delta endotoxin gene fused to a kanamycin
resistance gene in such a manner that both insecticidal
activity and kanamycin resistance are expressed.

A preferred embodiment is a recombinant construction of
Clavibacter xyli subsp. cynodontis ("Cxc") with Bacillus
thuringiensis subsp. kurstaki ("Cxc/Bt"). In a particularly
preferred embodiment, the Cxc harbors a chromosomally
integrated plasmid which contains the gene encoding Bt. In
another preferred embodiment, the plasmid encoding the Bt
gene "reverts" or segregates itself from the chromosome of
Cxc.

In another preferred embodiment of a Cxc/Bt recombinant
construct, the Cxc does not have the capacity to transfer the
introduced genetic material to other microbes.

In still another preferred embodiment, the Cxc/Bt recombinant declines rapidly in field soil. In a particularly preferred embodiment, the Cxc/Bt recombinant declines at the same rate of the wildtype microbe.

If not conducted in the initial screening of hybrid microorganisms, the hybrid organisms must, sooner or later, be screened directly for the ability to produce the agricultural chemical of interest. Preferably, the screening should be conducted at the earliest practical point in the process, which will be dictated by the number of hybrid microorganisms surviving at any particular stage of the process and the power of the technique used to screen for production of the agricultural chemical of interest to identify agricultural-chemical-producing hybrids out of a mixed inoculum.

It is to be understood that the screening for a particular agricultural-chemical-producing trait for use in accordance with the present invention will be within the capabilities of one having ordinary skill in the art through the exercise of routine experimentation in light of the teachings contained herein and inferences logically to be drawn from the disclosure of the present invention. The techniques contemplated as useful in screening for ability to produce the agricultural chemical may be determined by reference to standard compendia.

In general, these screening procedures will fall into one of four categories. These are (1) survival screens, where the agricultural chemical being produced is essential to survival of the hybrid and is not provided by the growth media; (2) analytical chemistry screens for the presence of the agricultural chemical being produced; (3) biological screens for the manifestations of biological activity associated with the agricultural chemical or chemicals being produced; and (4) immunoassay screens whereby organisms or groups of organisms producing a particular agricultural chemical are identified by interaction with an antibody which is specific to the chemical in question. The suitability of

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any particular screening technique or combination of
techniques will be dictated by the agricultural chemical in
question. Thus, survival screens are particularly useful in
identifying hybrid microorganisms capable of producing their
own fixed nitrogen by survival on nitrogen-deficient media.
Biological screens are particularly useful in identifying
hybrid microorganisms capable of producing antibacterial or
antifungal agents. A particularly preferred screening
technique of this sort involves layering a culture of
organisms susceptible to the antibiotic produced by the
hybrid microorganism over a mixed culture of hybrids and
identifying and removing for further screening hybrids or
groups of hybrid producing that antibiotic from those areas
where the susceptible organism cannot grow. Immunoassay
techniques are particularly appropriate where the
agricultural chemical of interest is a large molecule, such
as a polypeptide. In any event, biological screens for the
known effects of the agricultural chemical in question can be
conducted using discrete cultures of hybrid microorganisms
once the number of such cultures has been reduced to a
manageable level, e.g., 10^2 - 10^3 cultures, by preliminary
screens for other selectable traits associated with the
agricultural-chemical-producing microorganism. Thus, hybrids
producing insecticidal compounds, for example, could be
identified by the inability cf susceptible insects to survive
on a culture of the hybrid. Such screening has the
disadvantage of requiring screening of a larger number of
colonies and requiring a protracted period of time to
manifest results but is nonetheless capable of identifying
hybrids with the ability to produce the agricultural chemical
of interest.

     It is to be understood that, with appropriate changes in
the screening procedure, hybrids capable of fixing nitrogen
anaerobically, as is done in Rhizobium, or in the presence of
very small amounts of oxygen, as is done by microaerophilic
bacteria, can be prepared in accordance with the present
invention. Specifically, oxygen must be minimized or removed

in the nitrogen free growth medium during screening for
nitrogen-fixing ability.

If such anaerobic nitrogen-fixing hybrids are to enter
into endosymbiotic relationships with plant host, the hybrid
must also contain genetic material, such as that contained in
species of Rhizobium, which will cause the plant host to
create an oxygen free or low oxygen environment not normally
found in crop plants. For this reason utilization of aerobic
nitrogen-fixing bacteria is preferred.

In order to determine which of the final group of hybrid
microorganisms is capable of entering into endosymbiotic
relationship with the host plant whereby agricultural
chemicals produced by the hybrid may be utilized by the plant
as a supplement to agricultural chemicals otherwise
available, it is normally necessary to conduct preliminary
yield gain screening or the like. For nitrogen-fixing hybrid
bacteria produced by cell fusion, for example, this is
accomplished by growing the host plant in an environment
deficient in the agricultural chemical, e.g., fixed nitrogen,
and comparing the dry weight after an appropriate period of
growth, e.g., six weeks, with the dry weight of similar
plants in similar environment but which had been infected, as
by injection, with the stable fusion hybrid bacteria selected
by the foregoing method. In the case of agricultural-
chemical-producing bacteria that fix nitrogen, experience has
shown that testing of 5 to 7 of the best fusion hybrids will
result in identification of 3 or 4 stable fusion hybrids
capable of creating statistically significant yield gains in
the host plant growing in nitrogen deficient soil due, it is
believed, to the ability of the stable fusion hybrids to fix
atmospheric nitrogen aerobically and to enter into
endosymbiotic relationships with the host plant.

It is envisioned that the endosymbiotic agricultural-
chemical-producing microorganisms formed in accordance with
the present invention may be further modified by natural or
artificial genetic techniques to improve their performance as
sources of agricultural chemicals for the plant host.

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Specifically, the microorganisms may be modified to reduce their resistance to cold temperatures, thereby preventing unintended proliferation of the hybrid microorganisms from year to year. Similarly, the endosymbiotic hybrid microorganisms formed in accordance with the present invention may be modified to enhance their stability to drought, disease or other physiological stress. In this regard, the stability of the hybrid under stress conditions, and the ability to minimize the likelihood of spontaneous or forced reversion to a pathogenic state, is desired in a commercial microbiological product. In addition, the hybrid microorganisms could be modified to excrete the agricultural chemical. Ideally, the microorganism formed in accordance with the present invention should be modified so that they cannot grow outside the plant host, as by the selection of mutants requiring nutritional supplementation specifically or nearly specifically provided by the plant host in question. Techniques for genetic manipulation and mutant selection for strains of, for example, _Azotobacter_ are well known in the art and are envisioned as being suitable for effecting the above-described and similar genetic manipulations of the stable hybrids formed in accordance with the present invention.

The stable hybrid microorganisms having the ability to produce agricultural chemicals and of entering into an endosymbiotic relationship with a plant host formed in accordance with the present invention may be used to prepare agricultural products in many ways. For example, hybrids formed in accordance with the present invention may be injected into seedlings or used to form coated seed of the plant host involved by association of the hybrid with a biodegradable nutrient carrier material which is coated on the seed. Similarly, hybrids formed in accordance with the present invention may be used to form infected seed products by application of the microorganisms directly to the seed of host plants. Alternatively, an agricultural soil drench may be prepared by mixing microorganisms formed in accordance

with the present invention with water and other appropriate
drench constituents for application to the leaves, stem and
roots of growing host plants and surrounding soil by spraying
or the like.

It is to be understood that application of the teachings
of the present invention to a specific problem or environment
will be within the capabilities of one having ordinary skill
in the art in light of the teachings contained herein.
Examples of processes within the scope of the present
invention and the microbial products resulting therefrom
appear in the following examples.

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Example 1

Preparation of a Fusion Hybrid Bacterium From
Azotobacter vinelandii and Erwinia
stewartii Capable of Fixing Atmospheric Nitrogen
Aerobically and of Entering Into an Endosymbiotic
Relationship With Corn
</div>

Approximately 10^8 bacteria of the species Azotobacter
vinelandii known to be capable of fixing nitrogen aerobically
were selected. Approximately 10^8 bacteria of a strain of
Erwinia stewartii mutants known to be resistant to both
streptomycin and tetracycline were also selected. Fusion
hybrids of these two types of bacteria were formed by a
variant of the procedure of Weiss referred to above.

The bacteria growing in mid-log phase were treated with
lysozyme at a concentration of 100 micrograms per milliliter
and with DNA-ase at a concentration of 5 micrograms per
milliliter in media containing EDTA at a concentration of 1
millimolar and magnesium chloride at a concentration of 5
millimolar, having a pH of 7 and with 12% added sucrose.
After 10 minutes, protoplasts and spheroplasts of the
original bacteria were formed. The temperature was then
lowered to 0°C and the protoplast-forming solution was washed
out. The resulting protoplasts were placed in 12% sucrose
solution at 0°C. The solution was then supplemented with 40%
polyethylene glycol of molecular weight about 6,000 in order
to induce protoplast and spheroplast fusion for a period of

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10 to 15 minutes. The fused bacteria were spun down in a
centrifuge and resuspended in 12% sucrose plus a complete
growth medium for 4 hours at 0°C. The temperature was then
raised to 30°C and was held there for 2 hours, after which
the hybrid fusion products were transferred to complete
growth medium containing no additional sucrose for 2 hours.

The initial selection procedure was then conducted at a
temperature of about 25-30°C.

The hybrid fusion products were transferred and grown on
Burke's nitrogen-free medium as described in Carlson, et al.,
"Forced Association Between Higher Plant and Bacterial Cells
in Vitro." Nature, Vol. 22, No. 5482, p. 393-395 (1974). The
medium was nitrogen-free and contained added streptomycin at
a concentration of 100 micrograms per milliliter and
tetracycline at a concentration of 50 micrograms per
milliliter. Only those fusion products manifesting both the
ability to fix atmospheric nitrogen aerobically and
manifesting the streptomycin and tetracycline resistance
associated with the Erwinia stewartii strain originally
introduced survived on this medium after a growth period of
about 3 days.

Samples of the mixed fusion hybrids from this culture
were used to infect corn seedlings approximately 3 to 4 feet
in height by injection between the first and second node of
the stalk tissue. After 4 days the corn plants were
dissected into transverse sections to recover bacteria that
moved to the anterior (top) of the plant, since Erwinia
stewartii interacts with plant tissue during the initial
phase of infection by spreading throughout the vascular
system of the plant without being recognized or destroyed by
the plant's disease-response system. Those fusion hybrids
with the greatest facility to spread throughout the vascular
system of the corn plant were generally found between the
fifth and sixth node.

The fusion hybrid bacteria found between the fifth and
sixth node of the corn plant were cultured for 3 days on
Burke's nitrogen-free medium as described by Carlson, et al.,

<u>supra</u>. After 3 days, a mixed inoculant from this nitrogen-
free culture was again used to infect corn seedlings
approximately 3 to 4 feet in height by injection between the
first and second nods of the stalk tissue. After about 3
days the plant was transversely dissected as above described
to recover bacteria that moved to the anterior of the plant.
Again, those fusion hybrids with the greatest facility to
spread throughout the vascular system of the corn plant were
generally found between the fifth and sixth node.

The fusion hybrid bacteria recovered from this portion
of the corn plant were then grown as separate colonies on
Burke's nitrogen-free medium. Twenty-five of the most
vigorous colonies were selected, and each was used to infect
one of 25 corn plants by injection. After 3 weeks, those
colonies which did not result in any visible manifestation of
disease in the infected corn plants were selected. From
among these, seven colonies which manifested the most
vigorous growth on nitrogen-free media, the widest area of
spread throughout the plant and least manifestation of
disease symptoms were selected for preliminary yield testing
and were identified as Fusion #1 through Fusion #7.

In the preliminary yield test, flats (30cm x 46cm)
containing 15cm of washed sterile sand over 10cm of Perlite
were sown with 24 corn seeds at a uniform spacing. Flats
were maintained in the greenhouse (at approximately 22-27°C)
and were watered twice daily with deionized water. Starting
at 10 days (so as to exhaust the seed nitrogen) the seedlings
were watered three times a week with a nutrient solution.
One liter of solution was applied and allowed to drain
through. The solution was a modified Long Ashton mix that
contained nitrate ion at a concentration of 6 micromolar as
its sole nitrogen. The Long Ashton mix was also modified to
contain nitrate ion at concentrations of 6 and 0 millimolar
and used in comparative runs A and C below, respectively.
Experiments were run for six weeks, at which time the aerial
portions of the plants were harvested and dried for one week
at 95°C. Dry weight data was then collected. For flats

containing infected plants, the bacterial strains were
introduced by injection at 10 days (at the same time as
fertilization was initiated). Each analysis was done in
triplicate (three flats of 24 plants per treatment) and that
data were subjected to statistical analysis. Seven different
bacterial fusions were selected by multiple screens between
Erwinia stewartii and Azotobacter vinelandii (Fusion #1
through #7) as above described and used in this work. Actual
dry weight yield data per flat (average of the three flats)
is as follows:

Inoculation	NO_3 Level	Average Flat Dry Weight (grams)	Percentage Increase Over Control
A. None	6 millimolar	116.8	
B. None (Control Value)	6 micromolar	34.8	
C. None	None	19.2	
Fusion #1	6 micromolar	44.8	29
Fusion #2	6 micromolar	39.6	14
Fusion #3	6 micromolar	72.4	108
Fusion #4	6 micromolar	33.2	-5
Fusion #5	6 micromolar	54.8	57
Fusion #6	6 micromolar	25.2	-28
Fusion #7	6 micromolar	56.8	63

Results with Fusion numbers 1, 3, 5, and 7 are
statistically significant.

Uninoculated comparative run A contains the optimum
level of nitrate in Long Ashton nutrient solution and is
indicative of results to be expected with corn growing in
well nitrogen-fertilized soil. Comparative run C, to which
no nitrate was added, shows the yields which may be expected
based only on residual nitrate contained in the seed of the
corn. Comparative run B, which represents the control value
containing nitrogen at levels of about 6 micromolar, is
indicative of growth to be expected in poorly nitrogen-
fertilized soil. The reduction in dry weight associated with
fusion product number 6 is apparently due to non-visible

pathogenic effects, even though none of the plants manifested
visual symptoms of disease. The results of fusion product
number 4 are not statistically significant. The results of
fusion products 1, 3, 5, and 7 show that the process of the
present invention was able to produce at least four stable
fusion hybrid products capable of fixing atmospheric nitrogen
aerobically and capable of entering into endosymbiotic
relationships with corn whereby corn growing under nitrogen
stressed conditions in conjunction with the fusion products
of the present invention produced yields of from 29% to 108%
greater than a control without the bacteria formed in
accordance with the present invention.

<div align="center">Example 2

Preparation of a Fusion Hybrid Bacterium from
Azotobacter vinelandii and Agrobacterium
tumefaciens Capable of Fixing Atmospheric Nitrogen
Aerobically and of Entering Into an Endosymbiotic
Relationship with Sugar Beets</div>

Approximately 10^8 bacteria of the species Azotobacter
vinelandii were selected. Approximately 10^8 bacteria of a
strain of Agrobacterium tumefaciens mutants which are
resistant to both streptomycin and kanamycin were selected.
Protoplast fusion was effected between these bacteria as in
Example 1. The fused protoplasts were transferred to Burke's
nitrogen-free medium as described in Example 1 supplemented
with streptomycin at a concentration of 100 micrograms per
milliliter and kanamycin at a concentration of 50 micrograms
per milliliter. After 3 days, only those stable fusion
hybrids capable of both fixing atmospheric nitrogen
aerobically and possessing the genetic material from the
pathogenic bacteria associated with streptomycin and
kanamycin resistance survived.

A mixed inoculum from this culture was introduced into a
suspension culture of carrot tissue cells in the manner
described by A.G. Matthysse, et al., Physiological Plant
Pathology, 20, 27-33 and 21, 381-387 (1982), which is
specifically incorporated herein by reference in its

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entirety. After about 3 hours, the carrot cells and those
fusion hybrids capable of binding to carrot cells in a manner
similar to the initial interaction of <u>Agrobacterium</u>
<u>tumefaciens</u> with plant tissue cells during the initial phases
of infection were separated from unbound bacteria by washing
3 times in sterile, distilled water. The carrot cells with
adhering hybrid bacteria were macerated and washed again.
The resulting suspension was spun down in a centrifuge and
the resulting pellet, consisting of cell walls and adhering
hybrid bacteria, was placed on Burke's nitrogen-free medium
for 3 days.

The cell-binding method as above-described was repeated
and the fusion hybrids thus selected were grown as separate
colonies on nitrogen-free media. After 3 days of growth, 25
of the most vigorous colonies were selected.

Each colony was used to infect one of 25 sugar beet
seedlings 8 to 10 inches high by insertion of a pin, which
had been dipped into the colony, into the area of the
hypocotyle between the root and the plant. Those hybrid
colonies which did not form a gall or tumor in the sugar beet
seedling after 3 weeks were selected for further screening.

The roots of each infected sugar beet plant which did
not manifest any visible symptoms of disease after 3 weeks
were then dissected into a number of transverse sections.
The distance down the root of the plant to which the hybrid
bacteria had spread was noted. Five of the 25 stable fusion
hybrids were selected for preliminary yield testing based
upon the vigorousness of their growth on nitrogen-free media,
the absence of visible manifestations of disease associated
with their injection into the plant and the facility with
which they were able to spread throughout the plant tissue.
These five fusion hybrids, designated Fusion #1 through
Fusion #5 were subjected to preliminary yield testing as
follows:

Flats (30cm x 46cm) containing 15cm of washed sterile
sand over 10cm of Perlite were plated with 24 germinated
sugar beet seeds at a uniform spacing. Flats were maintained

in the greenhouse (at approximately 22-27°C) and were watered
twice daily with deionized water. Starting at 10 days (so as
to exhaust the seed nitrogen) the seedlings were watered
three times a week with a nutrient solution. One liter of
solution was applied and allowed to drain through. The
solution was a modified Long Ashton mix that contained
nitrate ion at a concentration of 6 micromolar as its sole
nitrogen. The Long Ashton mix was also modified to contain
nitrate ion at concentrations of 6 and 0 millimolar and used
in comparative runs A and C below, respectively.

Experiments were run for six weeks, at which time the
plants were harvested and dried for one week at 95°C. Dry
weight data was then collected. For flats containing
infected plants, the bacterial strains were introduced by
injection at 10 days (at the same time as fertilization was
initiated). Each analysis was done in triplicate (three
flats of 24 plants per treatment) and the data were subjected
to statistical analysis. Five different bacterial fusions
were selected by multiple screens between _Agrobacterium_
tumefaciens and _Azotobacter_ _vinelandii_ as above described and
used in this work. Actual dry weight yield data per flat
(average of the three flats) is as follows:

Inoculation	NO_3 Level	Average Flat Dry Weight (grams)	Percentage Increase Over Control
A. None	6 millimolar	44.7	-
B. None (control value)	6 micromolar	8.8	-
C. None	None	5.2	-
Fusion #1	6 micromolar	8.0	-9
Fusion #2	6 micromolar	12.4	41
Fusion #3	6 micromolar	6.9	-22
Fusion #4	6 micromolar	24.6	180
Fusion #5	6 micromolar	14.9	69

Results with Fusion numbers 2, 3, 4 and 5 are
statistically significant.

Uninoculated comparative runs A, B, and C have the same
significance as uninoculated comparative runs A, B, and C in
Example 1 above. Fusion product No. 3 appeared to result in
non-visible pathogenic effects, even though no visible
symptoms of disease were observed on any of the plants. The
preliminary yield data confirms that the process of the
present invention was capable of producing at least three
stable hybrid bacteria (Fusion #'s 2, 4 and 5) capable of
fixing atmospheric nitrogen aerobically and of entering into
an endosymbiotic relationship with sugar beets whereby sugar
beets growing in nitrogen stressed conditions generated
yields 41 to 180% in excess of those obtained with
uninoculated sugar beets.

Example 3

Example 2 was repeated with the exception that the
fusion products were applied to sugar beets receiving
nitrogen in the form of nitrate at a concentration of 6
millimolar -- that associated with optimal nitrogen
fertilization. No significant yield gain above that attained
by uninoculated comparative run A was observed in the
inoculated sugar beets. These results confirm that the yield
gains associated with the fusion products in Example 2 were
due to an endosymbiotic relationship between the fusion
products and the plant whereby fixed nitrogen was provided by
the fusion products and used by the plant host. Had the
yield gains reported in Example 2 been the result of growth
regulators or hormones provided by the fusion products,
similar gains should have been observed in Example 3.

Further evidence of an endosymbiotic association with
the host is provided by light microscopy using gram stain and
from reisolation of the bacteria (with the expressions of the
maintained antibiotic resistance markers) from infected
plants. Moreover, the hybrid bacteria can be found
throughout the plant. Their location is not limited to the
site of infection. The number of hybrid bacteria in plant
tissue was approximately 10^5 per gram (dry weight) in a corn
plant growing under low nitrate conditions.

It is to be understood that, with appropriate changes in the screening procedure, hybrids capable of fixing nitrogen anaerobically, as is done in Rhizobium, or in the presence of very small amounts of oxygen, as is done by microaerophilic bacteria, can be prepared in accordance with the present invention. Specifically, oxygen must be minimized or removed in the nitrogen-free growth medium during screening for nitrogen-fixing ability.

If such anaerobic nitrogen-fixing hybrids are to enter into endosymbotic relationships with plant hosts, the hybrid must also contain genetic material, such as that contained in species of Rhizobium, which will cause the plant host to create an oxygen free or low oxygen environment not normally found in crop plants. For this reason utilization of aerobic nitrogen-fixing bacteria is preferred.

Some of the fusion products of the prevent invention have also been observed to form spores. Selection of endosymbiotic bacteria formed in accordance with the present invention which form spores facilitates application and survival of the bacteria when applied to plant hosts since the spores are generally more resistant to stress than the growing bacterial cells.

Example 4

Production of a Fusion Hybrid Expressing Invertebrate Pesticide Activity for Use in Sweet Potatoes

The streptomycete S. ipomoea is a pathogen of Ipomoea species, which includes morning glories and sweet potatoes (Ipomoea batata). This organism invades both the fleshy root and fibrous roots of the latter, and is an endophyte candidate for fusion with an appropriate agchemical producing bacterium. Such a partner is S. avermitilis, which produces a family of compounds called avermectins, which are effective against a variety of insect pathogens, such as nematodes, mites, and insects. Campbell, W. C. et al., Science 221: 823-828 (1983). Using the technique of protoplast fusion, hybrids of S. ipomoea and S. avermitilis were produced, which

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can associate with sweet potato tissue and produce
avermectins.

Spores (10^8) of S. avermitilis strain SA-5 (nic) on
deposit with the American Type Culture Collection, 12301
Parklawn Dr., Rockville, Maryland 20852 USA under Accession
No. 53319 and S. ipomoea wild type SI-1 on deposit with the
American Type Culture Collection, 12301 Parklawn Dr.,
Rockville, Maryland 20852 USA under Accession No. 53320 were
inoculated into, respectively, 30 ml of YSG 1 and YSG 2 broth
(modified YSG 1 with 14% sucrose and 0.2% glycine). YSG 1
was prepared according to the method of Chater, K.F., et al.,
Cur. Top. Micro. Bio. Immunol. 96: 69-95 (1982), specifically
incorporated herein by reference in its entirety. Both
cultures were incubated at 30° with vigorous aeration (240
rpm) for 24 hours. Protoplasts were prepared from both
strains and suspended in protoplast buffer med P as described
in Hopwood, D.A., et al., Mol. Gen. Genet. 162: 307-317
(1978), specifically incorporated herein by reference in its
entirety. SI-1 protoplasts were irradiated with UV light at
254 nm (Model UVGL-58 Mineralight Lamp, UVP Inc., San
Gabriel, California) at 8 cm from the source for 2 minutes to
give 1% cell survival. UV-irradiated SI-1 protoplasts (10^7)
were mixed with 10^7 SA-5 protoplasts and sedimented by
centrifugation at 6000 rpm (Sorval SS-34 rotor) for 5 min at
4°C. The pelleted protoplasts were suspended gently in 0.1
ml of med P, and 0.9 ml of 40% (wt/vol) PEG 8000 (Sigma) in
med P was added followed by gentle mixing. After incubation
at 25°C for 3 minutes, the PEG-treated protoplasts were
diluted 10-fold with med P, centrifuged and suspended in 2 ml
of med P as previously. Samples (0.1 ml) of the fused
protoplasts were plated at 30°C onto protoplast regeneration
plates (RM16: yeast extract 0.2%, casamino acids 0.01%,
sucrose 15%, glucose 1%, oatmeal 0.3%, L-proline 0.1%,
$MgCl_2.2H_2O$ 1%, Na_2SO_4 0.025%, agar 2%, trace element
(Hopewood, D.A., op. cit.) 0.2%. After autoclaving the
following sterile solutions were added: $CaCl_2$ 20 mM; KH_2PO_4
5 mM, MES (2-(N-Morpholino) ethane sulfonic acid) buffer (pH

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6.5), 25 mM). The regenerated colonies were replica-plated
by cellulose acetate membrane filters (pore size 0.45um,
diameter 82 mm, Micro Filtration System, Japan) onto minimal
agar HMM plates (Hopwood, D. A., _Bacterial Rev._, 31: 373-403
(1967)) to screen for prototrophs. The latter were
subsequently patched onto YDC plates yeast extract 0.4%, malt
extract 1%, dextrose 0.4%, maltose 0.4%, agar 1.5%. After
autoclaving, $MgSO_4$ (10 mM) and CaCl 6.25 mM were added. The
plates were incubated for 7 days until the cells had
sporulated, and those with SI-1 colony morphology and
inability to produce melanin on YDC plates were further
screened for avermectin activity by a toxicity test using
freshly hatched brine shrimp. A 1 mm^2 piece of colony was
placed in the well of a 96-well microtiter dish (nunc) and
0.01 ml of methanol added. Brine shrimp (ca. 10 per 0.1 ml)
were added and the time of paralysis noted, and compared to
controls. Strain SI-1 gave no paralysis in this test
compared to control; strain SA-5 produced complete paralysis
in 15 minutes at 22°C.

Among 10^5 regenerated colonies, 270 showed SI colony
morphology. Ten percent of the latter exhibited avermectin
activity (paralysis within one hour), and 6 of these positive
clones were further inoculated onto sweet potato slices to
assay for their ability to associate and grow in plant
tissue. Moyer, J.W. et al., _Phyopathology_, 74: 494-497
(1984). All 6 grew on sweet potato slices, and 2 of these
recovered from the plant tissue retained avermectin activity.

<u>Example 5</u>

<u>Endophyte Hybridization with Recombinant DNA</u>

The bacterial species <u>Clavibacter xyli</u> subsp. <u>cynodontis</u>
(Cxc) and subsp. <u>xyli</u> (Cxx) are vascular inhibitors of
Bermuda grass and sugar cane, respectively, Davis, M.J. et
al., <u>Int. J. Syst. Bacteriol.</u>, 34: 107-117 (1984), which is
specifically incorporated herein by reference in its
entirety. They can grow in other commercially valuable crops
such as sorghum. Liao, C.H., et al., <u>Phytopathology</u>,
71:1303-1306 (1981), which is specifically incorporated

herein by reference in its entirety. It has been discovered that Cxc and Cxx can also grow in field and sweet corn without pathogenic effect. The following example teaches how the organism Cxc can be modified to produce an insecticidal protein, the Delta endotoxin of <u>Bacillus thuringienesis</u> var. Kurstaki HD-73, which is active upon lepidoptercus insects such as corn earworm (<u>Heliothus zea</u>) and European corn borer (<u>Pyrausta nubilalia</u>), which are pests of field and sweet corn.

<u>Preparation of Integration Vector</u>

The genes for agricultural chemicals are preferably carried on an integration vector for Cxc to prevent transmission of cloned genes to other organisms by plasmid transmission. Integration vectors have the foreign gene sequences inserted into or adjacent to a natural sequence of DNA from the organism whose genome is the integration target. See Saunders, C.W. et al., <u>J. Bacteriol</u>. 157: 718-726 (1984). The integration vector is commonly propagated in a permissive host such as <u>E. coli</u> or <u>B. subtilis.</u>

The manipulation of the foreign agricultural chemical gene sequences is carried out in <u>E. coli</u> or <u>B. subtilis</u> using replication vectors for these hosts. When the desired gene configuration is produced, the configuration is removed and cloned into the Cxc integration vector adjacent to or within the Cxc cloned DNA segment, and the integration vector is propagated in the permissive host. Purified integration vector DNA is then used to transform the desired Cxc host, selecting for a marker such as drug resistance, which is also carried in the foreign gene configuration. Since the vector does not carry a replication origin for propagation in Cxc, the vector must recombine with the Cxc chromosome in order to persist in Cxc. Recombination is facilitated by the Cxc homologous sequence on the integration vector. The stable drug-resistant transformants are then screened for the production of the agricultural chemical gene products, e.g. <u>B. thuringenesis</u> endotoxin.

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Recombinant DNA Methods

The restriction enzymes, T4 DNA ligase and calf
intestinal alkaline phosphatase, used in the following
methods were purchased from New England BioLabs, Bethesda
Research Laboratories, or Boehringer Mannheim. The reaction
conditions were those recommended by manufacturer.

Isolation of TBIA DNA

A 3 litre culture of Clavibacter xyli subsp. cynodontis
(TBIA, on deposit with the American Type Culture Collection,
12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under
Accession No. 33973) was grown aerobically in S8 medium
(Davis, M. J., et al., Science 210: 1365-1367 (1980)) to
mid-exponential stage at 28°C, at which point glucose and
glycine were added to final concentrations of 2% and 0.1%,
respectively. After growth for 17 hours, the cells were
centrifuged, washed with lysis buffer (50 mM glucose, 25 mM
Tris-Cl (pH 8.0), 10 mM EDTA) and resuspended in 2 ml of the
same. The cells were then lysed by addition of guanidine
hydrochloride and sarkosyl to final concentration of 7M and
4%, respectively, and Tris-Cl (pH 8.0) to 20 mM, and EDTA to
20 mM. Sung, M.T., et al., Genetic Engineering in the Plant
Sciences , N.J. Parapoulos (ed.), pgs. 39-62 (Praeger
Scientific Press 1981), specifically incorporated herein by
reference in its entirety. The final volume was 20 ml.
After overnight incubation at 50°C, 4 ml of distilled water
was added to the lysed suspension and the mixture spun at
12,000 rpm for 10 minutes at 20°C in the SS34 Beckman rotor.
This was done to separate cell wall and membrane debris from
the nucleic acids remaining in the supernatent. The
supernatent fluid was mixed with ethidium bromide (final
concentration equal to 600 ug/ml) and applied to a 2-step
cesium chloride gradient which was centrifuged for 24 hr.,
26000 rpm at 20°C in a Beckman SW27 rotor. The top step
consisted of 4.42 M CsCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA
to a final density of 1.55; the bottom step consisted of 5.7M
CsCl, 0.02M Tris-Cl (pH 8.0), to a final density of 1.7. The
DNA band was extracted and purified by standard techniques.

Maniatis, T., et al., <u>Molecular Cloning, A Laboratory Manual</u>
(Cold Spring Harbor Laboratory 1982), specifically
incorporated herein by reference in its entirety.
<u>TBIA Chromosomal Fragment Isolation</u>

TBIA chromosomal DNA was partially digested with the
restriction enzyme, Sau3Al, resulting in DNA fragments
ranging from approximately 1 kb to 25 kb (as shown by 0.7%
agarose gel electrophoresis). DNA fragments approximating 10
kb were isolated by centrifuging the partial Sau3Al digest of
TBIA DNA through a sucrose gradient. M. R. El-Gewely and R.
B. Helling, <u>Anal. Biochem.</u> 102:423-428 (1980).
<u>Cloning of TBIA DNA Fragment</u>

The cloning vector, pUC19 (New England Biolabs 1985-86
Cata-logue, p.90-91) was digested with the restriction
enzyme, BamHI, to create a linear plasmid with cohesive ends.
To prevent self-religation, the ends were treated with calf
intestinal alkaline phosphatase. Maniatis, T., et al., <u>op.</u>
<u>cit.</u> The Sau3Al digested TBIA fragments approximating 10 kb
(isolated by above procedure) were then ligated into the
unique BamHl site of pUC19 using T4-DNA ligase. The
concentrations of vector (pUC19) and insert (Sau3Al digested
TBIA fragments) used to obtain the correct circular
constructions were 0.0044 and 0.0156 ug per microliter of
ligation mix, respectively. Dugaiczyk, A., et al., <u>J. Mol.</u>
<u>Biol.</u> 96: 171 (1975); Helfman, D.M., et al., <u>Focus</u> vol. 6,
No. 1 (Bethesda Research Laboratories 1984). The ligation
was carried out with T4 DNA ligase at 0.05 units per
microliter of ligation mix at 14°C for 48 hours. The
ligation mixture was then transformed into competent <u>E. coli</u>
JM109 cells (on deposit with the American Type Culture
Collection, 12301 Parklawn Dr., Rockville, Maryland 20852 USA
under Accession No. 53323), using the calcium chloride
procedure. Maniatis, T., et al., <u>op. cit.</u> Transformants
with inserts were identified as ampicillin resistant white

colonies in the presence of Xgal. Vieira, J. and Messing, J.,
Gene 19: 259 (1982).

Screening of Transformants

Positive transformants (ampicillin resistant white
colonies) were screened for large TBIA DNA inserts (equal to
or greater than 10kb) which contained unique restriction
sites near or at the middle of the insert. Transformants
were lysed by the alkaline lysis plasmid DNA mini-prep method
(Maniatis, T., et al., op. cit.) and subjected to restriction
enzyme digests. A preferred plasmid from this screening was
pCG300 (on deposit with the American Type Culture Collection,
12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under
Accession No. 53329), which contains a 13 kb TBIA DNA insert
with one EcoR1 site near the middle of the insert (Fig. 1).

Cloning of Kanamycin Resistance Gene

In order to determine that the EcoRI site in the TBIA
insert of pCG300 is not located in a critical TBIA gene, a
kanamycin resistance cassette (Pharmacia 1984 Catalogue
Molecular Biologicals, Chemicals and Equipment for Molecular
Biology p. 74) was cloned into the site and this DNA
transformed into TBIA, selecting for kanamycin resistant
integrates. The recombinant plasmid, pCG300, was partially
digested with the restriction enzyme EcoRI, and ligated
(using T4 DNA ligase), with the EcoRI DNA fragment containing
the kanamycin resistance gene from Tn903. Dugaiczyk, A., et
al., J. Mol. Biol. 96:171 (1975); Pharmacia 1984 Catalogue
Molecular Biologicals, Chemicals and Equipment for Molecular
Biology p. 74. The ligation mixture was incubated at 14°C
for 72 hours. The ligation mixture was then transformed into
competent E.coli strain SK2267 (on deposit with the American
Type Culture Collection, 12301 Parklawn Drive, Rockville,
Maryland 20852 USA under Accession No. 53324); transformants
were selected on plates containing kanamycin sulfate (50 ug/
ml). Kanamycin resistant transformants were screened for the
favored construct -- the kanamycin gene cloned into the EcoRI
site found in the TBIA DNA insert of pCG300. The new
plasmid, pCG306 (Fig. 2), is then used to transform TBIA

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cells, selecting for kanamycin resistance. The finding of
stable transformants shows that the EcoR1 site can be used
for integration of agricultural chemical genes without
compromising the growth of TBlA.

Cloning of promoters from TBIA

    In order to insure that foreign genes will be expressed
in TBIA, it is preferable to clone regulatory sequences from
TBIA for fusion to foreign gene sequences.

    Sau3Al fragments from a partial restriction digest of
TBIA chromosomal DNA (see above) were cloned (Maniatis, T.,
et al., op. cit.) into the BamHI site of a Bacillus promoter
cloning plasmid, pPL703 (on deposit with the American Type
Culture Collection, 12301 Parklawn Dr., Rockville, Maryland
20852 U.S.A. under Accession No. 53327) which was derived
from pPL603. Williams D. et al., J. Bacteriol. 146: 1162-
1165 (1981). This plasmid (Fig. 3) contains a structural
gene for chloramphenicol acetyltransferase (CAT) which is not
expressed due to lack of a promoter. Recombinant plasmids
were transformed into competent Bacillus subtilis 62037 (rec
E) (on deposit with the American Type Culture Collection,
12301 Parklawn Dr., Rockville, Maryland 20852 U.S.A. under
Accession No. 53325). Restriction fragments that promote
expression of chloramphenicol acetyltransferase gene were
identified by selecting chloramphenicol resistant
transformants. Twenty-one chloramphenicol resistant colonies
were obtained, and their chloramphenicol acetyltransferase
activities were measured. Shaw W. V., in Methods in Enzymol.
43: 737-755 (Academic Press 1975). The transformants
contained recombinant plasmids with TBIA DNA insertions
ranging from 0.4 to 2 kilobases and chloramphenicol
acetyltranferase activity ranging from 49 to 1026 nmole/min/
mg protein. The plasmid pCG6 (Fig. 3) (on deposit with the
American Type Culture Collection, 12301 Parklawn Dr.,
Rockville, Maryland 20852 U.S.A. under Accession No. 53328)
with the highest promoter activity for chloramphenicol
acetyltransferase is the preferred source of regulatory
signals for the expression in TBIA of desired agricultural

chemical proteins, e.g. <u>Bacillus thuringiensis</u> insecticidal
endotoxin.

<u>Expression of B. thuringiensis Delta-Endotoxin
from a Cxc Promoter</u>

The M13 vector mBTK65 (on deposit with the American Type
Culture Collection, 12301 Parklawn Dr., Rockville, Maryland
20852 U.S.A. under Accession No. 53326) contains a truncated
<u>B. thuringiensis</u> delta-endotoxin gene fused to a kanamycin
resistance gene in such a manner that both insecticidal
activity and kanamycin resistance are expressed. The fusion
sequence is bounded by a BglII site 5-prime to the Shine-
Delgarno (ribosome binding site) sequence and a HindIII site
3-prime to the kanamycin resistance gene (Fig. 4). This
fusion hybrid sequence can be ligated 3-prime to a Cxc
promoter (as in pCG6) to bring expression of both activities
under Cxc control. The procedure is as follows: Phage
mBTK65 replicative form (RF) DNA is prepared from ATCC No.
53326 by standard methods. Barnes, W.M., et al., in <u>Methods
in Enzymology</u>, 101: 98-122 (Academic Press 1983). The
purified DNA is cut sequentially with the restriction enzymes
BglII and HindIII to liberate a 2.64 Kb fragment, which is
isolated and purified by preparative agarose electrophoresis
as described in Maniatis, T., et al., <u>op. cit</u>. The purified
fragment is then blunted by treating with the klenow fragment
of DNA polymerase as described in Maniatis, T., et al., <u>op.
cit.</u>

In order to optimize the translation of the delta
endotoxin-kanamycin resistance fusion gene, the promoter
segment of pCGC6 is treated with the exonuclease Bal-31 to
generate multiple 3-prime termini for fusion to the Shine-
Dalgarno sequence of the gene fusion. Roberts, T.M., et al.,
<u>Proc. Natl. Acad. Sci. USA</u>, 76: 760-764 (1979); Deans, R., et
al., in <u>Recombinant DNA Techniques</u>, 2:2-6 (The University of
Michigan 1981). In order to select kanamycin resistance
generated by the fusion hybrid, it is necessary to inactivate
the neomycin (kanamycin) resistance element already present
in pCG6. This is done by cutting pCG6 with BglII

endonuclease, which cute within the neomycin resistance gene;
the BglII ends are then filled in with the Klenow fragment of
DNA polymerase and the blunted vector is recircularized with
T4 ligase. Maniatis, T., et al., op. cit. The ligated DNA
is transformed into B. subtilis strain 62037 as before,
selecting chloramphenicol resistance and screening for
kanamycin sensitivity, indicating that a frameshift has
resulted in inactivation of the neomycin resistance element.
This plasmid is designated pCG6 Neo^S. Following digestion of
pCG6 Neo^S with SalI, the DNA is subjected to Bal-31 as
described (Deans, R., op. cit.) (Fig. 5) to generate a family
of deletions, whose pattern is monitored by gel
electrophoresis. The heterogeneous plasmid fragment
population is then ligated with the blunted BglII-HindIII
fragment, above. The ligated DNA is transformed into B.
subtilis 62037 (rec E), selecting for kanamycin resistance.
Since the expression of kanamycin resistance in the gene
fusion is dependent on the transcription and translation of
the 5-prime delta-endotoxin gene, only favorable
configurations of promoter and the delta-endotoxin gene
fusion will give rise to kanamycin resistance. The kanamycin
resistance transformants are screened by standard recombinant
DNA techniques (Maniatis, T., et al., op cit.) to verify the
restriction map. The expression of delta-endotoxin activity
is monitored by subjecting freshly-hatched larvae of the
tobacco hornworm (Carolina Biologicals) to samples of the
colony incorporated into their standard diet. Kanamycin
resistant transformants found to produce effective levels of
larvae toxicity are grown up and their plasmid DNA extracted.
The plasmid DNA is cut by SmaI and HindIII restriction
endonucleases which cut out the promoter-gene fusion region
(cf. Fig 5) and the ends blunted as before. This fragment is
then cloned into the integration vector pCG300 at the blunted
EcoRI site by standard methods (Maniatis, T., et al., op.
cit.). The ligated DNA is transformed as previously into E.
coli SK2267, selecting for kanamycin resistance.
Transformants from the latter procedure are screened as

before for insecticidal activity and expected restriction
map. Transformants giving the correct restriction map and
insecticidal activities are then grown up for plasmid DNA
preparation and subsequent transformation of TBIA.

Preparation of TBIA protoplasts

    TBIA protoplasts are prepared by the following method:
    1)   inoculate TBIA from frozen glycerol stock into S8
          medium;
    2)   aerate the culture at 30°C until mid-exponential;
    3)   add 2% glucose, 0.1% glycine to the culture, and
          continue aeration overnight (approximately 17
          hours);
    4)   spin down 20 ml of culture in sterile tubes at 8000
          rpm for 5 minutes at 4°C, wash once in 10 ml of
          SMMC buffer (sorbitol 0.5M - 20 mM maleate - 20 mM
          $MgCl_2$ - 20 mM $CaCl_2$ (pH 7.0) (Yoshihama M., et al.,
          J. Bacteriol. 162: 591-597 (1985)) and suspend in
          2 ml of SMMC buffer containing 2.5 mg/ml lysozyme;
    5)   incubate at 30°C for 2-3 hours, and check for the
          formation of protoplasts under a microscope; and
    6)   harvest cells by centrifugation at 6000 rpm for 10
          minutes at 4°C, wash once with 5 ml of SMMC buffer,
          and suspend in 1 ml of SMMC buffer.

Transformation of TBIA protoplasts

    A 20 ml exponential culture of TBIA growing in S8 medium
is pretreated with 2% glucose and 0.1% glycine overnight
(approximately 17 hours), and protoplasts are prepared as
described above. Samples (0.3 ml) of protoplasted cells are
placed in tubes, and the integrative plasmid DNA carrying the
TBIA promoter and B. thuringiensis delta endotoxin fusion
gene (1 ug DNA in 10 ul of 0.5 M sorbitol (pH 7.0)) is added.
Then 0.7 ml of 25% PEG in SMMC buffer is added, followed by
gentle mixing. After incubation at 25°C for 5 min., the
mixture is diluted 10 fold with SMMC, centrifuged as above,
suspended in 1 ml of SSC broth (S8 broth with 0.5 M sorbitol
(pH 7.0)), and incubated with shaking at 30°C for 2 hrs.

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The transformed protoplasts (0.1 ml) are plated onto cellulose acetate membrane filters on top of nonselective regeneration plates (SSC), and incubated for 2-3 days at 30°C to allow expression of kanamycin resistance. The filters are then transferred to selective regeneration plates (SSC + kanamycin (50 ug/ml)), and incubated further at 30°C to select for kanamycin resistant transformants.

Test for Larvacidal Activity of Hybrid TBIA in Corn

Kanamycin resistant transformants (above) are then grown in S8 medium plus 50 ug/ml kanamycin and portions of the culture tested for larvacidal activity _in vivo_ as described above. The larvacidal isolates are then inoculated into corn seedlings (FR 632, Illinois Foundation Seeds). Inoculations are performed by piercing 2 - 3 week old seedlings with a pointed sterile scapel which has been dipped in a colony to be tested. Periodically during the life cycle of the plant, xylem sap samples are removed and tested for kanamycin resistant hybrids which are larvacidal _in vivo._ As stated elsewhere in this document, isolates that routinely show colonization of the corn without disease symptoms, and which express the agricultural chemical _B. thuringiensis_ endotoxin) are selected for tests of _in vivo_ efficacy. The latter test is performed by challenging inoculated corn plants with freshly hatched larvae of the corn earworm or the European corn borer.

By way of further example of agricultural-chemical-producing microorganisms that can be used as sources of genetic material for use in the foregoing procedure, such microorganisms may contain genes encoding antibiotics synthesized by plants, invertebrates and vertebrates. These agricultural-chemical-producing microorganisms include those in which the agricultural chemical gene has been introduced by genetic engineering means. Non-limiting examples of these agricultural chemicals include lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, and magainins. Also included are animal and plant derived protease inhibitors. These inhibitors are known to increase plant defense

responses, or to enhance the insecticidal activity of
proteins such as those produced by <u>Bacillus thuringiensis</u>.
Trypsin inhibitors from the cowpea plant (<u>Vigna unguiculata</u>)
and other legumes are effective antimetabolites for control
of insects. A polypeptide isolated from tomato leaves, and
referred to as systemin, induces the synthesis of
wound-inducible protease inhibitors in tomato plants.

The genes encoding the agricultural chemicals discussed
above can be used according to the methods of the present
invention to transform an infecting microorganism. However,
these methods can be employed to transform an infecting
microorganism with a gene encoding any proteinaceous crop
protection agent produced by microorganisms, plants,
vertebrates, or invertebrates.

In this example, methods are described for expressing
genes encoding eukaryotic proteins as crop protection agents
in recombinant bacteria. The general methods for preparing
an integration vector and transfecting a microorganism are
described above in this example.

Plant chitinases are potent growth inhibitors of fungi
(Schlumbaum, A. et al., <u>Nature</u> <u>324</u>:365-367 (1986)). These
enzymes can be obtained from leaves of bean plants.

These antifungal proteins have also been identified in
radish seeds (Terras, F. R. G. et al., <u>J. Biol. Chem.</u>
<u>267</u>:15301-15309 (1992)). Antimicrobial peptides have been
isolated from the seeds of the four o'clock plant (<u>Mirabilis</u>
<u>jalapa</u> L.) (Cammue, B.P.A. et al., <u>J. Biol. Chem.</u>
<u>267</u>:2228-2233 (1992)). Other plant-derived enzymes useful as
crop protection agents include lysozyme and 1,3-glucanases.

Crop protection agents may also be obtained from
non-plant sources, such as vertebrates and invertebrates.
Zasloff has identified a class of antimicrobial peptides from
vertebrates, notably frog skin, referred to as magainins
(Zasloff, M., <u>Proc. Natl. Acad. Sci.</u> <u>84</u>:5449-5453 (1987);
Bevins, C. L. and Zasloff, M., <u>Ann. Rev. Biochem.</u> <u>59</u>:395-414
(1990)). These peptides each contain 23 amino acids, and at
low concentrations they inhibit the growth of numerous

species of bacteria and fungi, and induce osmotic lysis of
protozoa. The cDNAs of magainin peptides have been cloned,
and additional magainin-type molecules have been identified
and characterized. Another class of proteins having strong
anti-bacterial activity is the family of cecropins. Cecropin
molecules have been identified in many species of insects,
which are invertebrates. The amino acid sequences for major
cecropins are known and have been published (Boman, H. G. et
al., Ann. Rev. Microbiol. 41:103-126 (1987)). A class of
anti- bacterial proteins having a larger molecular weight
than cecropins has been identified, and these proteins are
referred to as attacins (Boman et al.). Attacins have been
identified in moth species.

An additional approach is to enhance the activity of
pesticidal proteins by use of protease inhibitors. Serine
protease inhibitors have been shown to enhance the activity
of delta-endotoxin proteins of Bacillus thuringiensis against
several important agricultural pests (MacIntosh, S.C. et al.,
J. Agricultural and Food Chemistry 38:1145-1152 (1990)).
Trypsin inhibitors from cowpea and other legumes are shown to
be effective antimetabolites active against, for example, a
beetle (Angharad, M.R. et al., J. Sci. Food Agric. 34:345-350
(1983)). The synthesis of defensive proteins in plant
tissues can be induced by a proteinase inhibitor (Pearce, G.
et al., Science 253:895-898 (1991)).

Additional agricultural chemical producing genes useful
in the present invention are those that encode enzymes active
in the synthesis of secondary metabolites. One such class
includes the polyketides and structurally related compounds
such as actinorhodin. Genes encoding the actinorhodin
synthetic pathway can be inserted into endophytes for the
control of plant fungal diseases, including rice blast.
Genes encoding enzymes for the synthesis of polyketide
antibiotics can be obtained from microorganisms.

However, microorganisms are not the only source of genes
for the production of agriculturally useful secondary
metabolites having antibiotic activity. The steroidal

compound squalamine, found in sharks, also demonstrates an
activity against E. coli, Staphlococcus, and Streptococcus.
See, Stone, R. in Science 259:1125 (1993). Anti-microbial
chemicals, termed phytoalexins, are produced by a wide
specturm of higher plants in response to infectious agents.
See, Robert N. Goodman, Zoltan Chiraly and K.R. Wood, in The
Biochemistry and Physiology Of Plant Disease, (U. Missouri
Press, Columbia, MO, 1986).

This example therefore also describes methods for
expressing genes encoding enzymes that synthesize secondary
metabolites in recombinant bacteria. The general methods for
preparing an integration vector and transfecting a
microorganism are described above in this example.

The molecular cloning of the whole biosynthetic pathway
of the Streptomyces antibiotic actinorhodin was first
described by Malpartida, F. et al., Nature 309:462-464
(1984). Malpartida et al. demonstrated that the genes
encoding the full biosynthetic pathway for actinorhodin can
be transferred between bacteria. A continuous segment of
Streptomyces coelicolor DNA carrying the complete genetic
information required for actinorhodin synthesis was
introduced into Streptomyces parvulus, and actinorhodin was
synthesized by the recombinant host.

Actinorhodin is synthesized from acetate units through
the polyketide pathway. Other polyketide antibiotics are
produced by fungi, actinomycetes, mycobacteria, and other
bacteria. (See, Hopwood, D. A. et al., Ann. Rev. Genet.
24:37-66 (1990).

In addition to naturally-occurring antibiotics, the
methods described herein are useful for producing hybrid
agricultural-chemical-producing microorganisms capable of
synthesizing novel hybrid antibiotics. Such methods are
known in the art, as exemplified by Hopwood, D. A. et al.,
Nature 314:642-644 (1985). Briefly, as a non-limiting
example, a plasmid containing the genes for the synthesis of
an antibiotic, such as actinorhodin, is inserted into a
microorganism producing a different but preferably

structurally related antibiotic, such as medermycin or
granacitin. Production of novel antibiotics can be detected,
for example, as alterations in the acid-base characteristics
of the culture relative to that of the wild-type organisms.
Routine screening of the novel compounds for antibiotic
activity will indicate recombinant hybrid antibiotic-
producing microorganisms that are useful for hybridization
with an infecting microorganism according to the methods of
this invention.

Genes from any prokaryotic or eukaryotic source encoding
any agricultural chemicals, or catalysts producing
agricultural chemicals, can be cloned into the Cxc
integration vector as described above in the example of
Bacillus thuringiensis endotoxin. It should be understood by
a person skilled in the art that agricultural chemicals can
include nucleic acids, proteins, lipids and carbohydrate
molecules, and their precursors. Stable transformants are
then screened for the production of the agricultural chemical
products.

EXAMPLE 6

CONSTRUCTION OF A CXC/BT MICROBE

This example sets forth (1) the construction a Cxc/Bt
microbe and (2) a verification of that structure. The test
microbe consists of a Maryland strain of Clavibacter xyli
subsp. cynodontis ("Cxc") that has been genetically
engineered to produce the delta endotoxin of Bacillus
thuringiensis subsp. kurstaki ("Bt") and has been designated
as MDR1.586.

MATERIALS AND METHODS

1.   Test Microbe

    A.   Construction of the Cxc plasmid integration
       vectors. The integration plasmid used to form
       MDR1.586 was pCG741.

       i.   Materials.

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The DNA segments comprising the integration vectors were obtained as follows:

1)   The E. coli replicon

The E. coli replicon pGEM 5Z (f+) was acquired commercially from Promega (Madison, WI). The colE1 origin of replication of this plasmid did not function in Cxc, nor did the ampicillin resistance gene.

2)   The homologous Cxc sequence

A chromosomal DNA fragment was isolated from Cxc which served as a site for homologous recombination, and which also provided a source for the promotion of mRNA synthesis in the vector, pCG741. The fragment was generated by the cloning of Sau3a fragments into the E. coli replicon pUC19 to produce a Cxc library.

3)   The delta-endotoxin gene

The delta-endotoxin gene of B. thuringiensis subsp. kurstaki HD73 was acquired from Dr. Arthur Aronson of Purdue University. The gene was cloned as a HinDIII fragment in an E. coli cloning vector. The complete sequence of the gene and flanking DNA is given in Adang, M.J.K., M.J. Staver, T.A. Rocheleau, J. Leighton, R.F. Barker and D.V. Thompson, 1985, Characterized full-length and truncated plasmid clones of the crystal protein of Bacillus thuringiensis subsp. kurstaki HD-73 and their toxicity to Manduca sexta, Gene 36:289-300. An abbreviated restriction map of the gene and flanking sequence is shown in Fig. 6. A portion of the sequence 5' to the N-terminus of the gene was modified by CGI with synthetic DNA. The relevant portion of the altered and native sequence are shown in Fig. 12. Functionally, CGI removed the Bt promoter region 5' to the natural leader transcription site, introduced several translation

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stop codons, and generated a polylinker region for directed DNA fusions.

4)   The tetracycline resistance marker

A 4.99 kb DNA fragment containing a tetracycline resistance determinant was obtained from the conjugative plasmid Tn916. This determinant has been designated tetM (Burdett, V; J. Inamine, S. Rajagopalan, 1982, Heterogeneity of tetracycline resistance determinants in streptococcus J. Bacteriol 149:995-1004) and operates at the level of protein synthesis (Burdett, V., 1987, Streptococcal tetracycline resistance mediated at the level of protein synthesis, J. Bacteriol 165:564-569). Homologues of this determinant, obtained from the transposon Tn1545 (Martin, P., P. Trieu-Cuot and P. Courvalin, 1986, Nucleotide sequence of the tetM tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tn1545 Nuc. Acids Res. 14) and from Ureaplasma urealyticum (Sanchez-Pescador, R., J.T. Brown, M. Roberts, and M.S. Urdea, The nucleotide sequence of the tetracycline resistance determinant tetM from Ureaplasma urealyticum, Nuc. Acids Res. 16) have been sequenced and shown to be 95% homologous at the amino acid level.

Analysis of the tetM gene of Tn916 shows it to be highly homologous to other sequenced tetM genes, and no functionality associated with transposition was found in this sequence.

The 16.4 kb transposon Tn916 was originally discovered on the chromosome of Enterococcus (Streptococcus) faecalis DS16 (Franke, A.E., and D.B. Clewell, 1981, Evidence for a chromosome-borne resistance transposon (Tn916) in Streptococcus faccalis that is capable of "conjugal" transfer in the absence of a conjugative plasmid J. Bacteriol. 145:494-502).

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The E. coli plasmid pAM120 was constructed by cloning a fragment of the Enterococcus plasmid pAD1 containing Tn916 into a pBR322 derived vector (Clewell, D.B. and C. Gawron-Burke, 1986, Conjugative transposons and the dissemination of antibiotic resistance in Streptococci, Ann. Rev. Microbiol. 40:635-659). The tetM gene has been previously mapped to a 4.9 kb HincII fragment (Fig. 8) within the transposon (Clewell, et al. 1986).

We used the 4.9 kb HincII tetM fragment (Fig. 3) for integration vector construction. This fragment, although derived from a conjugative transposon, is unlikely to possess the capacity for mobilization through either transposition or conjugation. This conclusion has been reached by a consideration of the sequence and by literature in which the functions of excision, transposition, and conjugal transmission have been genetically analyzed.

The conjugative transposon Tn916 is able to transpose to plasmids (e.g., pAD1 in E. faecalis, above) and to transfer to the chromosomes of strains of other Streptococcal species by conjugative functions encoded within the transposon. By "hitchhiking" on other conjugative plasmids and by conjugation, Tn916 has also been shown to transfer to other genera of bacteria, including some gram negatives (Clewell et al. 1986).

Senghas, et al. (Senghas, E., J.M. Jones, M. Yamamoto, C. Gawron-Burke, and D.B. Clewell, 1988, Genetic organization of the bacterial conjugative transposon Tn916, J. Bacteriol 170:245-249) carried out a study in which various functions Tn916 were insertionally inactivated by Tn5 mutagenisis. The result of this work was that the functions of excision, transposition and conjugative transfer

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via transposition were mapped to portions of the
transposon outside of the HincII fragment
containing the tetM gene. The majority of the
critical functions for conjugation mapped to the
right of the HincII tetM fragment (Figure 9).
Significantly, three insertional mutations
inactivating the excision function, which mapped at
the left end of Tn916 outside the tetM fragment,
could be complemented by the separately cloned end
showing the dissociation of this function from
tetM.

    Additional evidence for the functions
localized at the left end of Tn916 comes from the
genetic analysis of Tn1545. This is also a
conjugative transposon containing tetM, and shows
virtual identity to Tn916 at its extremities, which
are the sites of transpositional insertion
(Caillaud, F., and P. Courvalin, 1987, Nucleotide
sequence of the ends of the conjugative shuttle
transposon Tn154, Mol. Gen. Genet. 209:110-115;
Clewell, D.B., S.E. Flannagan, Y. Ike, J.M. Jones,
and C. Gawron-Burke, 1988, Sequence analysis of
termini of conjugative transposon Tn916
J. Bacteriol. 170:3046-3052). Poyart-Salmeron C.,
P. Trieu-Cuot, C. Carlier, and P. Courvalin, 1989,
Molecular characterization of two proteins involved
in the excision of the conjugative transposon
Tn1545 homologies with other site-specific
recombinases EMBO (Eur. Mol. Biol. Organ) 8:2425-
2434 have determined the sequence of the end of
Tn1545 corresponding to the left end of Tn916.
Here they found two open reading frames (ORF1 and
2) with substantial (35%) homology to the carboxy
half of the Pin protein of E. coli which functions
in site-specific recombination; these ORF's
corresponded to two proteins made in a cell-free
system. These functions were able to complement,

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in vivo, mutations which inactivated transposition
of Tn1545

These data have led to a model of
transposition of Tn916 and Tn1545 which predicts
that excision of the transposon is the first and
rate-limiting step of transposition, that the ORF2
protein is essential for that process, and that a
circular intermediate results which can then insert
into other sites (Clewell et al. 1986 and Poyart-
salmerson et al. 1989). Just such an intermediate
of Tn916 has been detected and purified, (Scoot, J.
R. P.A. Kircchman, and M.B. Caparon, 1988, _An_
intermediate in transposition of the conjugative
tranposon Tn916, Proc. Natl. Acad. Sci. 85:4809-
4813) and is able to transpose when introduced into
B. subtilis.

The HincII fragment containing the tetM gene
of Tn916 lacks both essential ends of the
transposon, including the ORF1 and 2 regions, plus
other critical functions to conjugation mapped by
Tn5 mutagenesis outside of this fragment. There
is, therefore, no basis to expect that this
fragment can excise, promote conjugation or
transpose itself or any element associated with it.

5)   Other DNA Segments

Several "polylinkers" of synthetic DNA were
employed to specifically join segments of the
integration vectors (see below).

ii.   Construction Sequence.

1.   Synthesis of pCG563, the basic
integration vector.

Fig. 10 illustrates the generation of
pCG563. The 4.8 kb tetM fragment was first
cloned into pGEMSZ(f+) to produce the
intermediate plasmid pCG560. The integration
sequence from pCG219 was cloned into pCG560 to
produce pCG563. This vector was capable of

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transforming MDE1 to Tcr when plated on 2 sg
tetracycline/ml of plate medium.

2.   Modification of the CryIA crystal protein
gene.

Fig. 4 illustrates the cloning and Fig.
12 the modification of the crystal protein
gene of <u>B. thuringiensis kurstaki</u> HD73. A
portion of Bt DNA was cloned in two steps from
the original vector FL15 to give the
intermediate vector pCG910. The fragment was
reduced in size by cloning the 3.77 kb Ndel
fragment into pGEMSZ(f+), to give pCG737. At
this point a synthetic polylinker was added at
the BstXl site to generate pCG738.

Removal of sequence 5' and 3' to the Ndel
sites has the following consequences: the <u>B.</u>
<u>thuringiensis</u> promoters I and II are removed
from the 5'end, and the second of two inverted
repeat stem-loops 3' to the gene is removed.
The addition of the polylinker creates
promoter cloning sites at the 5'end, as well
as translation stop codons. A comparison of
native and artificial DNA sequence at the 5'
end is shown in Fig. 12. All native Bt
sequence 5' to the NdeI site has been removed
and substituted, as shown.

3.   Construction of pCG741.

Fig. 13 shows the construction of the Bt
integration plasmid pCG741.

The plasmid pCG741, when transformed into
Cxc, produces the 130 kd toxin, plus
degradation products of the protein (see
Example 10). The source of promotion for
expression has been determined by S1 nuclease
mapping to be the Cxc DNA 5' to the ApaI site
if the Bt polylinker.

B.   Transformation of Cxc strain MDE1 with pCG741.
      i.   Materials
      1)   Cxc strain MDE1.
            Strain MDE1 is the wild-type Maryland isolate.
      2)   DNA.
            Plasmid DNA was prepared from E. coli by
            equilibrium density centrifugation according
            to standard techniques (Maniatis et al.)
      ii.   Methods
      1)   Preparation of cell samples.
            Cultures of MDE1 were grown in S27 medium at
30°C to late-log phase. The cells were harvested
by centrifugation in the cold (4°C) and washed with
ice-cold water. The final cell suspension was
brought up in 10% sucrose to a concentration of ca.
1010 cells/40 ul.
      2)   Electroporation.
            Plasmid DNA was added to 40 ul samples of the
cell suspension and transferred to a prechilled
electroporation cell (Bio-Rad). Electroporation
took place in the Bio-Rad Gene Pulser. The cells
were then diluted with S27 medium at 22°C and
plated on cellulose acetate filters atop SC plates.
After 18 hr at 30°C the filters were transferred to
SC plates containing 2 ug tetrlcycline/ml.
      3)   Growth of transformants.
            After approximately 10 days Tcr colonies
appeared. The transformants were purified and
their Bt expression determined. A representative
Cxc transformant containing pCG741 was designated
MDR1.586.
2.   Test Microbe Characteristics
      A suspension of Cxc strain MDR1.586 or MDE1 in water is
a yellow odorless liquid, density approximating water. The
pH is approximately 6. The cells are 0.2-0.3 u in diameter
and 1-2 ul in length. A typical suspension contains 10^9-10^{10}
per ml. The cells, when frozen at -20°C, are stable for at

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least one month, or a year if glycerol is added to a final
concentration of 50%.

3.   Method for Verifying Integration

Plasmid pCG741 integrated into the Cxc chromosome by
crossover within the region of DNA homology defined by the
Cxc chromosomal fragment. This recombination converted the
circular plasmid to a linear form within the Cxc chromosome.
To detect this conversion of the circular plasmid to a linear
insert, the technique of Southern hybridization was used to
map the borders of the integrated DNA. The overall
structural integrity of the insert was determined by
comparing restriction fragment mobilities of the inserted DNA
with that of the pure plasmid. The presence of border
fragments - fragments with one restriction site within the
plasmid and the other site within the Cxc genome beyond the
region of homology - was used to show both the fact of
chromosomal integration as well as the site of integration.

4.   Test System

The test system used for this study was restriction site
analysis of DNA isolated from the test microbe, MDR1.586.

ANALYTICAL METHODS

Total DNA from MDR1.586 was purified and cut with
specific restriction endonucleases. DNA fragments were
separated by agarose gel electrophoresis, and the pattern of
restriction fragments was determined by hybridization with
specific radiolabelled DNA probes:

       a.   Source of Probes (Fig. 14).
       Bt gene: the 600 bp EcoRI fragment.
       pGEM 5Z (f+): the complete 3 kb plasmid.
       TetM: the 1.7 kb XbaI - HindIII fragment.
       Cxc integration sequence: the 5kb XbaI-EcoR1
       fragment.
       b.   Restriction Digests.
       In order to determine whether integration
       occurred, the restriction enzyme SfiI was used
       which cut pCG741 once in a non-Cxc portion of the
       plasmid (Fig. 15). Cleavage at this site allows an

unintegrated plasmid to be detected as the linear fragment. If the plasmid is integrated, the plasmid Sfil site would generate two new fragments from Sfil sites in the genome outside the plasmid boundaries (Fig. 15).

In order to assess the integrity of the integrated plasmid, the restriction enzyme Kpn1 was chosen. This enzyme has four sites in pCG741, one of which is in the Cxc portion of the plasmid (Fig. 15), and the fragments generated correspond to the four different elements of the plasmid. If the plasmid integrates by a Campbell-type crossover event which generates a duplication of the Cxc integration sequence (Fig. 15), Kpn1 will generate a circularly permuted map of the integrated plasmid DNA indistinguishable from the unintegrated plasmid. This digest generates the fragments shown in Fig. 15.

The patterns were visualized by autoradiography and their mobilities determined by reference to DNA molecular weight standards.

1.   Isolation of DNA.

DNA was isolated from Cxc strains MDE1 and MDR1.586 by adaptations of the standard cleared-lysate procedure (Maniatis, T., E.F. Fritsch and J. Sambrook, 1982, Molecular Cloning, a Laboratory Manual, Cold Spring Laboratory, N.Y. (hereinafter "Maniatis, et al., 1982")). Lysozyme treated cells were disrupted with sodium dodecyl sulfate (SDS) and treated with proteinase K. After chloroform/phenol extractions the DNA was repeatedly precipitated from ethanol, and resuspended in TE buffer (0.01 M Tris-HCl, pH 8.0, 0.005 M Na-EDTA).

2.   Restriction Enzyme Analysis.

DNA samples were digested with the indicated restriction enzymes according to manufacturers' instructions.

The digested samples were electrophoresed in agarose gels of indicated concentration in TAE buffer (Maniatis,

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et. al., 1982) for lengths of time appropriate to resolve
fragments of the expected size.

3.   Transfer.

Following ethidium bromide staining and photography, the
DNA fragments were denatured with base, neutralized, and
transferred from the gels to "Gene-Screen Plus" membranes
(New England Nuclear Research Products) according to the
manufacturer's instructions. The transfer was done using a
vacuum apparatus (Vacu-Blot, ABN products) according to
manufacturer's instructions.

4.   Hybridization.

Hybridization probes were prepared from the indicated
restriction fragments resolved on agarose gels by standard
techniques (Maniatis, et al., 1982). The fragments were
labeled with 32P-alpha dCTP by the "oligo labelling"
technique with the appropriate kit (Boehringer).

Hybridization of the labeled probe with the membrane-
bound , DNA fragments was carried out according to
instructions provided by the manufacturer of the membrane
(New England Nuclear). The gels were hybridized with the
indicated probes in a solution containing 1 M NaCl, 1% SDS
(sodium dodecyl sulfate) and 10% dextran sulfate at 65°C for
18 hrs. The membranes were washed twice with (a) 2 x SSC
(0.3 M NaCl, 0.03 M Na Citrate) for 5 min, 21 C; (b) 2 x SSC
plus 1% SDS at 65 C for 30 min; (c) 0.1 x SSC at 21 C for 30
min. The membranes were autoradiographed at 22 C for varying
lengths of time to provide a clear image.

Mobilities of fragments were determined by reference to
stained molecular weight standards.

RESULTS

Autoradiographic data from Southern hybridizations:

Fig. 16 shows the result of SfiI digests of
DNA samples, specifically probing for Cxc DNA
homologous to the integration sequence. Lane 5
(strain MDE1 DNA) shows a 16.2 kb hybridizing
fragment, corresponding to the region of the native
genome which contains the DNA of the integration

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vector. Lane 1 shows the uncut, and lane 2 the
Sfil digested plasmid pCG741 alone. The latter 17
kb fragment corresponds to the position of linear
plasmid. Lane 3 shows the digest of an artificial
mixture of pCG741 and MDE1 DNA. The native Cxc
Sfil fragment and the linear plasmid migrated
similarly, and form a doublet. Lane 4 shows the
Sfil digest of strain MDR1.586. Both the native
genome fragment and the linear plasmid band are
missing, replaced by two new fragments (22.8 and
11.7 kb) which sum (34.5 kb) reflects the sum of
the two they replaced (33.2). These data show that
the plasmid has been converted to a linear form
integrated into the chromosome of Cxc, at the site
of homology corresponding to the integration
sequence of the plasmid.

Fig. 17 shows the result of Kpn1 digests of
DNA probed with probes homologous to different
portions of the integration plasmid pCG741. Each
subset is the identical digest probed with a
different probe. The data show that the integrated
plasmid in strain MDR1.586 (lane 4) gives the same
bands as an artificial mixture (lane 3) of the pure
plasmid (lane 2) and MDE1 DNA (lane 5); these data
show that the integrated plasmid has not been
rearranged or deleted, within the sensitivity of
the technique.

The amount of plasmid DNA (5 ng) in the
artificial mixture of MDE1 (2 ug) and plasmid was
computed to be equal to a frequency of one copy per
genome (ca. 4000 kb). The similar hybridization of
the plasmid signal to that of the homologous DNA
fragment in the recombinant MDR1.586 shows that
there is approximately one integrated plasmid per
genome.

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CONCLUSIONS

The results of the Southern analysis demonstrated that:

1.   The restriction fragment map of pCG741 and the identity of the different components of the integrated plasmid in MDE1 chromosome were the same. The plasmid did not undergo detectable rearrangements following integration.

2.   A restriction enzyme digest fragment of the wild-type Cxc chromosome (MDE1), the target site of integration, disappeared as expected in the recombinants. This demonstrated that its linear continuity had been altered. Two new fragments appeared in the recombinant, corresponding to the two border fragments expected at the junction of the integrated linear plasmid and the target site on the Cxc chromosome. Integration relied on homologous recombination between the cloned segment of chromosomal DNA in pCG741 and the chromosomal site in MDE1. The plasmid was inserted in its entirety with no detectable rearrangements.

3.   No unintegrated plasmid was detected. Plasmid CG741 does not exist in an unintegrated form in Cxc.

4.   The autoradiographic intensity of the hybridized integrated sequences was equivalent to the expected intensity of a single copy of the relevant sequence diluted in the Cxc genome. The copy number of the integrated plasmid is similar to that of the genome.

EXAMPLE 7

IN PLANTA LABORATORY AND GREENHOUSE BIOASSAYS
OF CXC/BT CONSTRUCTIONS AGAINST EUROPEAN CORN BORER

The objective of this study is to determine the ability of a Cxc/Bt recombinant strain to prevent or reduce damage to corn caused by artificial infestations of the European corn borer (ECB), Ostrinia nubilalis (Hubner), in the greenhouse.

MATERIALS/METHODS

Test Microbe

The test microbe used in the experiments reported here was Crop Genetics strain MDR1.586 (genotype pCG741/MDE1; phenotype TetR SC+Tet; prepared as in Example 6 above). The wild-type Maryland isolate of Cxc (MDE1) was used as an endophyte control. Cells were stored in 50% glycerol at -20xC or in 2% BSA at -80xC. Viability checks were performed on both strains prior to use in each experiment (Table 3).

Test System

The test system for this study is field corn. The European corn borer was chosen as the test insect because it is the primary target pest for Cxc/Bt.

Test Site

Plant lot IP89-3 (field corn variety PD 003) was planted July 20, 1989, and inoculated August 12 with either Cxc/Bt recombinant or wild-type Cxc suspended in PBS, or with sterile PBS, using the stab inoculation method. Plant lot IP89-4 (also variety PD 003) was planted August 17 and inoculated August 30. Plant lot IP89-5 (varieties PD 003 and PD 093) was planted October 6 and inoculated October 23. The strain numbers for test microbes used in these experiments are presented in Table 3.

Each experiment was arranged in a split-plot design with individual greenhouse benches as main plots and treatment groups as sub-plots arranged in rows, the positions of which were randomized within each bench. Experiment IP89-3/GH-1 occupied 3 benches, each bench containing 7 plants per treatment group for a total of 21 plants per treatment. Experiment IP89-4/GH-1 also occupied 3 benches, but each treatment was represented by 30 plants (10 per bench). Experiment IP89-5/GH-1 occupied 4 benches, with 24 plants per treatment (6 per bench) for each of 2 corn varieties (PD 003 and PD 093). Experiment IP89-5/GH-2 also occupied 4 benches,

with 24 plants per treatment (6 per bench) for both corn
varieties.

Inoculation

Inoculating devices were prepared by sharpening the
eye end of No. 18 tapestry sewing needles. Needle volume
was determined gravimetrically by the amount of water held in
the eye. Only needles which held 3 ul (+/- 10%) were used.
The needles were mounted in chucked metal handles, and the
whole apparatus autoclaved before use. Inoculum was
introduced into the plants at approximately 10 - 20 cm above
the soil line by dipping the needle in a suspension of the
test microbe or wild-type Cxc to fill the eye, then stabbing
with the needle eye through to the center of the stem. The
needle was then carefully withdrawn to ensure that the
inoculum had been retained in the plant. Each plant was
stabbed twice from different angles to insure it received
sufficient inoculum. The dose was calculated to be between
approximately 5×10^6 and 8×10^7 CFU per plant (Table 3).
Procedures for inoculating plants are provided in co-pending
United States Patent Application Serial No. 07/368,167, filed
June 6, 1989, to Jed Fahey, entitled "Delivery of Beneficial
Microorganisms to Seeds and Plants."

Test Method

Only plants confirmed as colonized by Cxc or Cxc/Bt
(except PBS controls) were infested with insects. Details of
the assessment of colonization incidence are presented below
under "Phase Contrast Microscopy." The numbers of plants
given above for each experiment (see "Test Site") include
only colonized, infested plants.

Plants were infested with European corn borers 5 to
6 weeks after inoculation, depending on incidence of
colonization. Experiment IP89-3/GH-1 was infested on
September 18, 37 days after inoculation. Experiment IP89-4/
GH-1 was infested October 16, 47 days after inoculation.
Experiment IP89-5/GH-1 was infested December 4, 42 days after
inoculation. Experiment IP89-5/GH-2 was infested December 5,
43 days after inoculation.

Experiments IP89-3/GH-1, IP89-4/GH-1, and IP89-5/
GH-1 were infested by drilling a 1/4-inch hole about 3/4 of
the way through the stalk in 5 internodes per plant,
beginning with the lowest internode that was easily
accessible. The drill bit was surface disinfested between
treatment groups by dipping it in 95% ethanol and flaming.
Each drill hole was infested with 3 neonate corn borer larvae
from egg masses obtained from French Agricultural Research
Service (Lamberton, MN). Egg masses were held at 10xC in a
Percival chamber for 7 days to synchronize development, then
transferred to another Percival chamber at 27xC for
approximately 48 hours for incubation and eclosion. Larvae
were transferred into the stem holes using camel hair
brushes, and the holes were then plugged with nonabsorbent
cotton. Experiment IP89-5/GH-2 was infested by placing 10
neonate ECB larvae into each of 5 leaf axils per plant using
a camel hair brush. This technique provided a more realistic
simulation of a natural infestation. The source and storage
conditions of egg masses were the same as in the other
experiments.

Sampling/Observations

All plants were assessed for damage due to corn
borer feeding 3 to 4 weeks after infestation. The dates for
each experiment were: IP89-3/GH-1, October 9 and 10; IP89-4/
GH-1, November 6 and 7; IP89-5/GH-1, January 2, 1990; IP89-5/
GH-2, January 3 and 4, 1990). Each plant was cut down at the
soil line and stripped of its leaves, which were examined for
evidence of larval feeding. The stalk was then split
longitudinally to expose corn borer feeding tunnels and any
larvae present. Ears were also dissected to expose tunnels
and insects. Data recorded for each plant included the
length of each tunnel, the weight and developmental stage
(instar) of each live insect recovered, and the instar of any
dead insects found in or on the plant.

ANALYTICAL METHODS

Description of Viability Procedure

Inoculum for greenhouse experiments was prepared
from 5-to 8-day-old cultures of Cxc or Cxc/Bt grown on solid
media (SC for Cxc isolate MDE1, SC + Tet2 for Cxc/Bt strains
MDR1.586). Cell mass was collected from the surface of the
medium with a sterilized rubber policeman and suspended in
PBS to a weight ratio of approximately 0.05g per ml. The
resulting concentration of viable cells was between 109 and
1011 CFU/ml when diluted and plated on solid media for
verification (see Table 3).

Calculations

Tunnel lengths were summed to yield total tunnel
length for each plant, providing an estimate of the total
amount of damage caused by corn borer feeding. Weights of
surviving insects were summed to yield total insect biomass
per plant, a composite measure that incorporates both
survival and growth of the insects.

Phase Contrast Microscopy

Phase contrast microscopy was used to determine
incidence of colonization of plants inoculated with Cxc and
Cxc/Bt strains. The lowest leaf not showing an inoculation
scar was removed from each plant, and sap was expressed from
the midrib onto a microscope slide. The drop of sap was
examined under oil immersion at 1000X. The plant was scored
as positive if at least 2 Cxc cells were observed within 30
seconds of viewing a single field. If fewer than 2 Cxc cells
were observed, or if there was uncertainty about the sample,
the plant was scored as negative. The selection criterion of
2 cells per field corresponds to approximately 107 CFU/ml
sap.

Colonization incidence sampling began at least 4
weeks after inoculation, and if necessary it was repeated
weekly thereafter (up to 6 weeks after inoculation) until at
least 20 plants in each inoculation treatment group were
confirmed colonized.

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Statistical Methods

     For Experiments IP89-3/GH-1, IP89-4/GH-1, and IP89-5/GH1, number of tunnels and total tunnel length per plant, number of live insects recovered per plant, and the total corn borer biomass recovered per plant (the sum of individual weights of insects from each plant) were analyzed by split-plot analysis of variance (alpha = 0.05), with benches as main plots and treatment groups as subplots. Treatment means were separated using Duncan's multiple range test at alpha = 0.05. For Experiment IP89-5/GH-2, these data were analyzed by split-split-plot ANOVA (alpha = 0.05), with benches as mainplots, corn hybrids as subplots, and treatment groups as sub-subplots. For all experiments, unbalanced data (average tunnel length, average weights and instars of live insects) were summarized as means and their standard errors, but were not subjected to ANOVA. All analyses and calculations of descriptive statistics were performed using the MSTAT statistical software (Nissen, O., E.H. Everson, S.P. Eisensmith, V. Smail, J. Anderson, K. Rorick, G. Portice, D. Rittersdorf, P. Wolberg, M. Weber, R. Freed, B. Bricker, T. Heath, and J. Tohme. 1985. User's guide to MSTAT (version 4.0). Michigan State University, East Lansing)

RESULTS/DISCUSSION

     Inoculation of corn plants with Cxc/Bt resulted in significant reduction in feeding damage caused by European corn borer larvae. In most cases, significant effects of Cxc/Bt on corn borer survival, growth, and development were also observed.

Experiment IP89-3/GH-1

     The number of borer tunnels in plants inoculated with Cxc/Bt strain MDR1.586 was reduced to one third of the number of tunnels found in either control group, while total tunnel length was reduced by more than 70% (Table 4).

     Only 14 to 15 percent of the larvae placed into the stalks of control plants were recovered alive at the end of the experiment. High larval mortality is common in European corn borer populations (Showers, W.B., and G.L. Reed, 1972,

methods of infesting with corn borer, Iowa State J. Sci.
46:429-434; Showers, W.B., M.B. DeRozari, G.L. Reed, and R.H.
Shaw, 1978, Temperature-related climatic effects on
survivorship of the European corn borer, Environ. Entomol.
7:717-723; Showers, W.B., J.F. Witkowski, C.E. Mason, D.D.
Calvin, R.A. Higgins, and G.P. Dively, 1989, European corn
borer: development and management, N. Central Regional
Extension Publ. No. 327. Iowa State Univ., Ames, Iowa.;
Siegel, J.P., J.V. Maddox, and W.G. Ruesink, 1987,
Survivorship of the European corn borer, Ostrinia nubilalis
(Hubner) (Lepidoptera: Pyralidaee in Central Illinois,
Environ. Entomol. 16:1071-1075) even when larvae are placed
directly into the relatively protective and nutritious
environment of the stem (Chiang, H.C., 1959, Survival of
European corn borer larvae, Pyrausta nubilalis (Hubn.), in
artificial tunnels in stalks of field corn, Zea mays, L. Ann.
Entomol. Soc. Am. 52: 631-632). By comparison, only about 6%
of the larvae placed in plants inoculated with Cxc/Bt strain
MDR1.586 were recovered alive at the end of the experiment.
Total corn borer biomass was reduced by nearly 2/3 (relative
to controls) in plants inoculated with Cxc/ Bt.
Experiment IP89-4/GH-1

Cxc/Bt strain MDR1.586 caused significant reduction
in both the number of corn borer tunnels per plant and the
total amount of feeding (total tunnel length) (Table 6).
Significantly fewer live insects were recovered from Cxc/Bt-
inoculated plants than from controls (a reduction of about
75%). Total corn borer biomass and average weight were also
less for insects recovered from the Cxc/Bt-inoculated plants
(Table 7).

Measurements of both plant damage and insect
survival and growth were lower than those obtained
previously, probably due to environmental changes (lower
temperatures and changing photoperiod) associated with the
changing seasons. Although the greenhouse was equipped with
heaters and supplemental lighting, it was impossible to
duplicate during the late fall and winter the conditions of

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high temperature and relative humidity (as well as maximal
solar illumination) that existed when the study began in the
summer. As a result, insect feeding and growth rates, which
are influenced strongly by environmental conditions
(particularly temperature), tended to be reduced during the
winter months.

Experiment IP89-5/GH-1

        Damage due to corn borer feeding was significantly
less in plants inoculated with Cxc/Bt recombinant strain
MDR1.586 than in plants inoculated with wild-type Cxc or
sterile buffer. The magnitude of this effect was greater in
corn hybrid PD 003 than in hybrid PD 093. In PD 093, total
tunnel length was reduced by about 50%, number of tunnels by
about 40%, and average tunnel length by about 15%, relative
to controls. The corresponding approximate percent
reductions for hybrid PD 003 were 80%, 55%, and 60% (Table
8). MDR1.586 caused a significant reduction in the number
and weight of surviving insects, but the magnitude of this
reduction was again greater for hybrid PD 003 than for hybrid
PD 093 (85% vs. 60% reduction in number of live insects per
plant and 80% vs. 30% reduction in average weight per insect,
relative to controls) (Table 9). In both control groups
(sham-inoculated and wild-type Cxc), plants of hybrid PD 003
tended to suffer more damage (total tunnel length) and
produce more ECB biomass (i.e. more and larger insects) than
did plants of hybrid PD 093. However, the two hybrids did
not differ significantly (except for total tunnel length)
when inoculated with MDR1.586 (Tables 8 and 9).

Experiment IP89-5/GH-2

        Corn plants inoculated with Cxc/Bt recombinant
strain MDR1.586 suffered less damage than did control plants,
despite heavy infestation pressure from externally-applied
corn borers. Cxc/Bt-inoculated plants contained fewer ECB
tunnels and less internal damage (measured as total tunnel
length) than did control plants (Table 10). As in previous
experiments, fewer and smaller live insects were recovered
from Cxc/Bt-inoculated plants than from controls (Table 11).

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Plants of hybrid PD 003 contained fewer tunnels, less
internal damage, and fewer, smaller larvae than plants of
hybrid PD 093, irrespective of inoculation treatment.
However, the relative effect of Cxc/Bt strain MDR1.586 on
these measurements was similar for both hybrids (i.e., the
main effects of hybrids and treatment were highly significant
by ANOVA, but their interaction was not).

CONCLUSION

The results of these experiments demonstrate that
Cxc/Bt strain MDR1.586 is capable of preventing or limiting
damage to inoculated corn caused by European corn borer under
conditions of artificial infestation in the greenhouse.
Experiments IP89-3/GH-1, IP89-4/GH-1, and IP89-5/GH-1
demonstrated that MDR1.586 produces sufficient amounts of BT
endotoxin to affect larvae feeding in the relatively
protected but highly Cxc/Bt-colonized stem. Experiment IP89-
5/GH-2 demonstrated that this activity is sufficient to
reduce feeding, growth, survival, and overall damage even
when insects were applied to external plant surfaces to
simulate more realistically a natural infestation by second-
generation ECB.

Table 3

Viability of test microbes used in study 40ENT.

	log CFU per ml inoculum			log CFU/plant (approx.)		
Strain Experiment:	IP89-3	IP89-4	IP89-5	IP89-3	IP89-4	IP89-5
MDE1	10.36	10.31	9.34	7.8	7.8	6.8
MDR1.586	10.36	9.26	9.60	7.8	6.7	7.1

Table 4

Measurements of plant damage, Experiment IP89-3/GH1. Data are means and
standard errors (N=21 plants per treatment). Corn variety was PD093.

Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PBS	Sham inoc. control	15.0 ± 2.5 a	3.7 ± 0.6 a	4.3 ± 0.4
MDE1	Wild-type Cxc control	13.1 ± 1.8 a	3.6 ± 0.6 a	3.8 ± 0.4
MDR1.586	Cxc/Bt recombinant	3.7 ± 1.1 b	1.3 ± 0.4 b	2.8 ± 0.3

Means within the same column followed by the same letter are not significantly different (Duncan's
Multiple Range Test, alpha = 0.05).

Table 5

Measurements of surviving European corn borers, Experiment IP89-3/GH-1. Data are means and standard errors. Corn variety was PD093.

Treatment	Description	Live insects recovered			Total ECB biomass per plant (mg)	Average Wt. (mg)	Avg. Instar
		Total	%	No./plant			
PBS	Sham inoc. control	48	15.2	2.3 ± 0.4 a	127 ± 25 a	53 ± 6	4.4 ± 0.1
MDE1	Wild-type Cxc control	44	14.0	2.1 ± 0.3 a	141 ± 22 a	67 ± 6	4.8 ± 0.1
MDR1.586	Cxc/Bt recombinant	18	5.7	0.9 ± 0.3 b	36 ± 13 b	42 ± 7	4.4 ± 0.2

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 6

Measurements of plant damage, Experiment IP89-4/GH-1. Data are means and
standard errors (N=30 plants per treatment). Corn variety was PD093.

Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PBS	Sham inoc. control	7.1 ± 1.0 a	2.5 ± 0.3 a	2.8 ± 0.2
MDE1	Wild-type Cxc control	8.6 ± 1.1 a	2.7 ± 0.3 a	3.2 ± 0.2
MDR1.586	Cxc/Bt recombinant	1.2 ± 0.4 b	0.8 ± 0.3 b	1.5 ± 0.1

Means within the same column followed by the same letter are not significantly different
(Duncan's Multiple Range Test, alpha = 0.05).

Table 7

Measurements of surviving European corn borers, Experiment IP89-4/GH-1. Data are means and standard errors. Corn variety was PD093.

Treatment Instar	Description	Live insects recovered Total	%	No./plant	Total ECB biomass per plant (mg)	Average Wt. (mg)	Avg.
PBS	Sham inoc. control	47	10.4	1.6 ± 0.2 a	62 ± 12 a	40 ± 4	4.6 ± 0.1
MDE1	Wild-type Cxc control	50	11.1	1.7 ± 0.2 a	96 ± 17 b	57 ± 5	4.7 ± 0.1
MDR1.586	Cxc/Bt recombinant	12	2.7	0.4 ± 0.2 b	7 ± 4 c	18 ± 5	4.3 ± 0.2

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 8

Measurements of plant damage, Experiment IP89-5/GH-1. Data are means and standard errors (N=24 plants per treatment).

Corn Hybrid	Treatment	Description	Total tunnel length/plant (cm)	Number of tunnels/plant	Average tunnel length (cm)
PD093	PBS	Sham inoc. control	20.4 ± 1.7 a	6.7 ± 0.4 a	3.0 ± 0.2
	MDE1	Wild-type Cxc	16.4 ± 1.8 b	5.2 ± 0.4 b	3.1 ± 0.2
	MDR1.586	Cxc/Bt recombinant	9.3 ± 1.4 c	3.6 ± 0.5 c	2.6 ± 0.2
PD003	PBS	Sham inoc. control	24.2 ± 1.9 a	6.1 ± 0.5 ab	3.9 ± 0.2
	MDE1	Wild-type Cxc	23.6 ± 2.6 a	5.7 ± 0.5 ab	4.1 ± 0.2
	MDR1.586	Cxc/Bt recombinant	4.6 ± 0.6 d	2.7 ± 0.4 c	1.7 ± 0.2

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 9

Measurements of surviving European corn borers, Experiment IP89-5/GH-1. Data are means and standard errors.

Corn Hybrid	Treatment	Description	Live insects recovered		No./plant	Total ECB biomass per plant (mg)	Average Wt. (mg)	Avg. Instar
			Total	%				
PD093	PBs	Sham inoc. control	86	24.9	3.7 ± 0.3 b	296 ± 30 b	79 ± 4	5.1 ± 0.1
	MDE1	Wild-type Cxc	68	18.9	2.8 ± 0.3 c	216 ± 30 c	78 ± 4	5.1 ± 0.1
	MDR1.586	Cxc/Bt recombinant	28	7.8	1.2 ± 0.2 d	62 ± 14 d	57 ± 8	4.7 ± 0.1
PD003	PBs	Sham inoc. control	113	31.4	4.7 ± 0.4 a	419 ± 40 a	89 ± 3	5.0 ± 0.1
	MDE1	Wild-type Cxc	104	30.1	4.5 ± 0.5 a	429 ± 52 a	95 ± 4	5.2 ± 0.1
	MDR1.586	Cxc/Bt recombinant	18	5.0	0.7 ± 0.1 d	15 ± 5 d	20 ± 6	3.7 ± 0.1

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 10

Tunneling damage caused by European corn borers applied as neonates to leaf axils, Experiment IP89-5/GH-2. Data are means and standard errors from 24 plants per hybrid and treatment.

Corn Hybrid	Treatment	Description	Total tunnel length/plant (cm)		Number of tunnels/plant		Average tunnel length (cm)
PD093	PBS	Sham inoc. control	34.9 ± 3.5	a	10.9 ± 1.0	a	3.3 ± 0.2
	MDE1	Wild-type Cxc	34.8 ± 2.8	a	10.4 ± 0.8	a	3.4 ± 0.1
	MDR1.586	Cxc/Bt recombinant	18.4 ± 2.1	c	6.7 ± 0.6	b	2.7 ± 0.2
PD003	PBS	Sham inoc. control	25.8 ± 2.1	b	8.3 ± 0.7	b	3.2 ± 0.2
	MDE1	Wild-type Cxc	20.8 ± 2.1	bc	7.7 ± 0.6	b	2.6 ± 0.2
	MDR1.586	Cxc/Bt recombinant	9.5 ± 1.1	d	4.0 ± 0.4	c	2.5 ± 0.3

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

Table 11

Measurements of surviving European corn borers, Experiment IP89-5/GH-2. Data are means and standard errors.

| Corn Hybrid | Treatment | Description | Live insects recovered | | | Total BCB biomass per plant (mg) | | Average Wt. (mg) | | Avg. Instar |
			Total	%	No./plant					
PD093	PBs	Sham inoc. control	207	17.3	8.6 ± 0.9	a	708 ± 80	a	82 ± 3	4.9 ± 0.03
	MDE1	Wild-type Cxc	195	16.0	8.1 ± 0.8	a	717 ± 75	a	88 ± 3	5.0 ± 0.03
	MDR1.586	Cxc/Bt recombinant	105	8.8	4.4 ± 0.4	c	281 ± 27	c	64 ± 4	4.8 ± 0.1
PD003	PBs	Sham inoc. control	174	14.5	7.3 ± 0.7	ab	532 ± 55	b	73 ± 3	4.8 ± 0.04
	MDE1	Wild-type Cxc	151	12.6	6.3 ± 0.6	b	498 ± 55	b	79 ± 4	4.9 ± 0.1
	MDR1.586	Cxc/Bt recombinant	68	5.7	2.8 ± 0.3	c	154 ± 22	c	54 ± 4	3.7 ± 0.1

Means within the same column followed by the same letter are not significantly different (Duncan's Multiple Range Test, alpha = 0.05).

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EXAMPLE 8

HOST RANGE OF CXC/BT CONSTRUCT

This example compares the survival and systemic
distribution of a new Cxc/Bt construction, set forth above in
Example 6, with that of an older, extensively tested
construction and wild-type Cxc in 6 different plant species.
These species appear to serve as differential hosts,
exhibiting a range of colonization levels.

MATERIALS AND METHODS

Test Microbes:

The first control strain utilized in this experiment,
strain MDE1, is a typical wild-type Cxc isolate. It was
cultured from a lot which had been stored as 50% glycerol
stocks at -20°C. This strain shows characteristic colony and
cell morphology when cultured on SC media and SCM media, no
growth on NBY (nutrient broth yeast extract agar), and a
positive reaction to anti-Cxx antisera.

The second control strain, MDR1.3, was derived from MDE1
and contains a Bt gene, a kanamycin resistance gene, and a
tetracycline resistance gene. This strain was cultured from
EPS lot 1 which had been stored as 50% glycerol stocks at -
20°C. This strain shows characteristic colony and cell
morphology when cultured on SC media and SCM media, no growth
on NBY media, and a positive reaction to anti-Cxx antisera.

As set forth above in Example 6, the test strain,
MDR1.586, was derived from MDE1 and contains a Bt gene and a
gene for tetracycline resistance. This strain was cultured
from a lot which had been stored as a 50% glycerol stock at -
80°C. This strain shows characteristic colony and cell
morphology when cultured on SC and SCM, no growth on NBY, and
a positive reaction to anti-Cxx antisera.

Test System:

The test system used in this experiment consisted of six
different plant species grown in 4" pots. Test species were
(1) redroot pigweed (Amaranthus retroflexus), (2) velvetleaf
(Abutilon theophrasti), (3) ivyleaf morningglory (Impomoea

hederaceae), (4) jimsonweed (Datura stramonium), (5) ragweed
(Ambrosia artemisiifolia), and (6) chive (Allium
schoenoprasum).

<u>Inoculation:</u>

Cells were scraped from the surface of 6-8 day old plate
cultures and suspended in sterile phosphate-buffered saline
at a ratio of 1 g cells:1000 ml PBS. This suspension was
inoculated into the plants approximately 2-3 cm above the
soil line. The inoculating device was the sharpened eye of a
#7 hand sewing needle held in a handle. At inoculation, the
morningglory was 19 days old, the pigweed and jimsonweed were
26 days old, the ragweed and velvetleaf were 33 days old, and
the chive was 40 days old.

The calculated inoculum dose for control strain 1 (MDE1)
was 1.9 x 106 CFU/g, for control strain 2 (MDR1.3) was 2.1 x
106 CFU/g, and for the test strain (MDR1.586) was 2.4 x 106
CFU/g.

<u>Test Method:</u>

Inoculated plants were randomly arranged on the
greenhouse bench in a split-plot design with plant species as
the main plots and strains as subplots. There were 6 plant
species, 3 strains, and 10 replications.

<u>Sampling:</u>

Samples were collected over a 5 day period (2
replications per day) from 27 to 31 days post-inoculation.
Plant parts assayed were: (1) a basal shoot section
(including the inoculation wound site if visible), and (2) a
distal sample taken from at least 10 cm above the basal
sample. Distal samples from pigweed, morningglory, and chive
were pooled from 3 branches from the equivalent region.
Samples were assayed on the day of collection.

ANALYTICAL METHODS

<u>Inoculum Viability Procedure:</u>

The inoculum suspension was serially diluted in
phosphate-buffered saline, and 0.1 ml droplets of the
appropriate dilutions were placed on SC medium. Counts were
made after 8 days incubation at 28 + 3C in the dark. The

dilution which gave counts the closest to between 8 and 80 colonies per droplet was counted.

<u>Culture Method:</u>

    1.   Disinfestation

        a.   Samples were washed of any adhering dirt with a weak detergent solution.

        b.   Samples were surface disinfested by immersion (with some agitation) in a solution of 10% bleach and 1 drop/ 1 Tween 80 for two minutes.

        c.   Samples were rinsed twice by immersion in sterile distilled water changed at appropriate intervals, and blotted dry on sterile paper towels. A minimum of about 5 mm of tissue was aseptically removed from the cut ends to eliminate any disinfestant which may have been absorbed by the tissue.

    2.   Plating

        a.   Thin slices of the sample were aseptically weighed and adjusted to the range of 0.1 to 1.0 grams.

        b.   The sample was aseptically transferred to a 25 x 150 mm capped sterile tube with 10 ml sterile PBS, and homogenized for 10-20 seconds with a Brinkman Polytron homogenizer equipped with a PTA 20 generator (or the equivalent).

        c.   Samples were placed in an ice water bath for 10-60 minutes to allow bacteria to dissociate from plant tissue.

        d.   After dissociation the sample was vortexed and large particles were allowed to settle, and a serial 10-fold dilution was made in sterile phosphate buffered saline.

        e.   Droplets (0.1 ml) of the appropriate dilutions were placed on duplicate plates of SCM.

        f.   Plates were incubated in the dark at 28 + 3C for 7-12 days before counting colonies.

    3.   Reading Plate Cultures

        a.   The dilution which gave counts the closest to between 8 and 80 colonies per droplet was counted.

    b.   A sample was considered contaminated if contaminants obscured any of the dilutions and no Cxc or Cxc/ Bt was seen.

    c.   A sample was recorded as "0" if Cxc or Cxc/Bt was not detected and counts were not obscured by contaminants.

Calculations:

    1.   Inoculum Dose

Colony forming units per ml of the inoculum suspension (CFU/ml) was calculated as follows:

        average count x drop factor x dilution factor = CFU/ml Where:

        - average count is the average number of colonies on all plates at the dilution counted;
        - drop factor is the inverse of the drop volume;
        - dilution factor is the inverse of the dilution.
        Needle volume was determined gravimetrically.
        Inoculum dose was calculated as follows:
        CFU/ml inoculum suspension x needle volume in ml = CFU inoculated.

    2.   Determination of CFU/g Sample

        The basic formula to obtain counts in colony forming units per gram sample (CFU/g) is as follows:

$$\frac{(av\ count)(drop\ factor)(homogenizing\ volume)(dilution\ factor)}{fresh\ weight\ in\ grams} = CFU/g$$

Where:

- av count is the average number of colonies on all plates at
  the dilution counted;
- homogenizing volume is the volume of PBS in the homogenate;
- drop factor is the inverse of the drop volume;
- dilution factor is the inverse of dilution.

3.   Log Transformation

Counts of bacteria are expressed as log10 (CFU/g). Because log10 0 is undefined, if CFU/g = 0, then log10 1 is used.

<u>Statistical Methods:</u>

Each plant species x plant part combination was subjected to single factor analysis for strain differences in populations of test microbes using SAS PROC GLM. Contaminated, missing, or 0 population samples were not included in the analysis. Within each of these groupings, strain means were compared using Duncan's Multiple Range at alpha = .05. Plant parts were also analyzed by SAS PROC GLM for strain differences in colonization incidence of test microbes over all plant species.

<div align="center">RESULTS AND DISCUSSION</div>

Population data are presented in Table 12. Individual analysis of variance for each species x part combination showed no difference in population levels between strains in 10 cases out of 11. In the basal samples from chives, populations of the recombinant strains were significantly higher than the wild type. Distal samples from jimsonweed could not be analyzed due to the low number of colonized samples.

Incidence of colonization for each plant species x plant part x strain combination is presented in Table 13. Although the number of values is too low for a meaningful analysis of strain differences in each individual plant species x plant part combination, analysis over all species showed no significant strain differences in either the basal or the distal samples.

Careful examination of the data reveals some differences from previous experiments. Population levels and incidence of colonization in morningglory, jimsonweed, ragweed, and chive were higher than in previous experiments. Populations in distal samples of pigweed and velvetleaf were slightly lower than in previous experiments. Previous experiments had been performed in the field or in the greenhouse during

summer months. In most cases, the inoculation to harvest interval was longer than 4 weeks, and the sample size was smaller. Differences in growth conditions and inoculation to harvest interval would be expected to influence populations. Larger sample size and variation in detection limits would be expected to influence incidence of colonization.

CONCLUSION

There was very little difference in population levels and incidence of colonization between the test strain MDR1.586 and the two control strains.

Table 12

Populations of new construction MDR1.586 and control
strains in basal and distal samples of various plant
species measured 4 weeks after inoculation.

Plant Species	Basal Samples			Distal Samples		
	Strain	n[1]	CFU/g[2]	Strain	n[1]	CFU/g
Pigweed	MDR1.3	9	5.72 a	MDR1.3	5	4.81 a
	MDR1.586	8	5.36 a	MDE1	5	4.74 a
	MDE1	9	4.96 a	MDR1.586	7	3.76 a
Velvetleaf	MDR1.586	9	7.42 a	MDE1	8	7.37 a
	MDR1.3	9	7.38 a	MDR1.3	9	7.09 a
	MDE1	10	7.38 a	MDR1.586	7	6.80 a
Morningglory	MDE1	8	4.60 a	MDR1.3	1	3.29 a
	MDR1.3	7	4.22 a	MDE1	3	2.79 a
	MDR1.586	9	4.07 a	MDR1.586	3	2.54 a
Jimsonweed	MDR1.586	8	4.13 a	MDE1	1	3.98
	MDR1.3	6	3.57 a	MDE1.3	1	2.57
	MDE1	3	3.24 a	MDR1.586	1	2.37
Ragweed	MDR1.586	5	4.85 a	MDR1.3	1	5.31 a
	MDR1.3	7	4.33 a	MDE1.586	5	4.61 a
	MDE1	4	3.61 a	MDE1	1	4.59 a
Chive	MDR1.3	3	7.19 a	MDE1	2	3.79 a
	MDR1.586	3	6.58 a	MDR1.586	2	3.44 a
	MDE1	2	2.77	MDR1.3	0	na[3]

[1]   Number of colonized samples.

[2]   Mean $\log_{10}$ colony forming units per gram sample tissue of
colonized samples. In each group, means followed by the same
letter not significantly different by Duncan's Multiple Range
Test (alpha = .05).

[3]   Not applicable.

Table 13

    Incidence of colonization of new construction MDR1.586
    and control strains in basal and distal samples of
    various plant species measured 4 weeks after
    inoculation.

Plant Species	Strain	Basal pos/post+neg[1]	Distal pos/post+neg
Pigweed	MDE1	9/9	5/10
	MDR1.3	9/9	5/7
	MDR1.586	8/9	7/9
Velvetleaf	MDE1	10/10	8/10
	MDR1.3	9/9	9/9
	MDR1.586	9/9	7/9
Morningglory	MDE1	8/9	3/10
	MDR1.3	7/9	1/9
	MDR1.586	9/10	3/10
Jimsonweed	MDE1	3/8	1/10
	MDR1.3	6/8	1/10
	MDR1.586	8/9	1/10
Ragweed	MDE1	4/7	1/7
	MDR1.3	7/8	1/5
	MDR1.586	5/5	5/8
Chive	MDE1	2/9	2/10
	MDR1.3	3/8	0/8
	MDR1.586	3/8	2/10

[1]   Number of colonized samples per number of observations.

EXAMPLE 9

DISTRIBUTION OF CXC/BT CONSTRUCT IN CORN

MATERIALS AND METHODS

Test Microbes:

The first control strain utilized in this experiment, strain MDE1, is a typical wild-type Cxc isolate. It was cultured from a lot which had been stored as 50% glycerol stocks at -20°C. This strain shows characteristic colony and cell morphology when cultured on GCab, SC and SCM, no growth on NBY, and a positive reaction to anti-Cxx antisera.

The second control strain, MDR1.3, was derived from MDE1 and contains a Bt gene, a kanamycin resistance gene, and a tetracycline resistance gene. This strain was cultured from a lot which had been stored as 50% glycerol stocks at -20°C. This strain shows characteristic colony and cell morphology when cultured on GCab, SC and SCM, as well as on these same media with tetracycline, no growth on NBY, and a positive reaction to anti-Cxx antisera.

As set forth above in Example 6, the test strain, MDR1.586, was derived from MDE1 and contains a Bt gene and a gene for tetracycline resistance. This strain was cultured from a lot which had been stored as a 50% glycerol stock at -80°C. This strain shows characteristic colony and cell morphology when cultured on GCab, SC and SCM, as well as on these same media with tetracycline, no growth on NBY, and a positive reaction to anti-Cxx antisera.

Test System:

The test system used in this experiment was a hybrid corn variety grown in 1 gallon pots in the containment greenhouse.

Inoculation:

Cells were scraped from the surface of 3-7 day old plate cultures and suspended in sterile phosphate-buffered saline at a ratio of 1 g cells: 1000 ml PBS. This suspension was inoculated into the shoot of 10 day old corn seedlings approximately 2-3 cm above the soil line. The inoculating device was the sharpened eye of a #18 or #20 hand sewing

needle held in a handle. The calculated inoculum dose was
1.7 x 107 CFU for MDE1, 9.8 x 106 CFU for MDR1.3, and 1.2 x
107 CFU for MDR1.586.

Test Method:

    Inoculated plants were randomly arranged on the
greenhouse bench in a split plot design with harvest dates as
the main plots and strains as the subplots. There were three
harvest dates, three strains, and five replications.

Sampling:

    Samples were collected on dates approximately 2 weeks,
6 weeks, and 10 weeks post-inoculation. On the 2 week
sampling date, plant parts assayed were: (1) the basal
shoot, and (2) the highest leaf with a visible leaf collar.
On the 6 and 10 week sampling dates, plant parts were: (1)
the basal internode, (2) the base of the ear leaf
(approximated if necessary as leaf #8 or #9), and (3) the
sub-ear internode. Samples were assayed on the day of
collection.

                        ANALYTICAL METHODS

Description of Viability Procedure:

    The inoculum suspension was serially diluted in
phosphate-buffered saline, and 0.1 ml droplets of the
appropriate dilutions were placed on SC medium. Counts were
made after 8 days incubation at 28 + 3C in the dark. The
dilution which gave counts the closest to between 8 and 80
colonies per droplet was counted.

Culture Method:

    1.   Disinfestation

        a.   Samples were washed of any adhering dirt with
a weak detergent solution.

        b.   Samples were surface disinfested by immersion
(with some agitation) in a solution of 10% bleach in water
with 1 drop/l Tween 80 for two minutes.

        c.   Samples were rinsed twice by immersion in
sterile distilled water changed at appropriate intervals, and
blotted dry on sterile paper towels. A minimum of about 5 mm
of tissue was aseptically removed from the cut ends to

eliminate any disinfestant which may have been absorbed by
the tissue.

    2.   Plating

        a.   Thin slices of the sample were aseptically
weighed and adjusted to the range of 0.1 to 1.0 grams.

        b.   The sample was aseptically transferred to a 25
x 150 mm capped sterile tube with 10 ml sterile PBS, and
homogenized for 10-20 seconds with a Brinkman Polytron
homogenizer equipped with a PTA 20 generator (or the
equivalent).

        c.   Samples were placed in an ice water bath for
10-60 minutes to allow bacteria to dissociate from plant
tissue.

        d.   After dissociation the sample was vortexed and
large particles were allowed to settle, and a serial 10-fold
dilution was made in sterile phosphate buffered saline.

        e.   Droplets (0.1 ml) of the appropriate dilutions
were placed on duplicate plates of GCab or SCM and SC +
tetracycline. Samples from MDE1-inoculated plants were
plated only on GCab or SCM.

        f.   Plates were incubated in the dark at 28 + 3C
for 7-12 days before counting colonies.

    3.   Reading Plate Cultures

        a.   Counts were obtained from the GCab or SCM
plates for all samples if possible. Counts were obtained
from the SC + tet plates only if counts could not be made
from the GCab or SCM plates.

        b.   The dilution which gave counts the closest to
between 8 and 80 colonies per droplet was counted.

        c.   A sample was considered contaminated if
contaminants obscured any of the dilutions and no Cxc or Cxc/
Bt was seen.

        d.   A sample was recorded as "0" if Cxc or Cxc/Bt
was not detected and counts were not obscured by
contaminants.

Calculations:

1.   Inoculum Dose

Colony forming units per ml of the inoculum suspension (CFU/ml) was calculated as follows:

average count x drop factor x dilution factor = CFU/ml

Where:

- average count is the average number of colonies on all plates at the dilution counted
- drop factor is the inverse of the drop volume
- dilution factor is the inverse of the dilution.

Needle volume was determined gravimetrically and rounded to the nearest ul.

Inoculum dose was calculated as follows:

CFU/ml inoculum suspension x needle volume in ml

= CFU inoculated.

2.   Determination of CFU/g Sample

The basic formula to obtain counts in colony forming units per gram sample (CFU/g) is as follows:

$$\frac{(av\ count)(drop\ factor)(homogenizing\ volume)(dilution\ factor)}{fresh\ weight\ in\ grams} = CFU/g$$

Where:

- av count is the average number of colonies on all plates at the dilution counted;
- homogenizing volume is the volume of PBS in the homogenate;
- drop factor is the inverse of the drop volume;
- dilution factor is the inverse of dilution.

3.   Log Transformation

Counts of bacteria are expressed as $\log 10$ (CFU/g). Because $\log 10\ 0$ is undefined, if CFU/g = 0, then $\log 10\ 1$ is used.

Statistical Methods:

Each harvest date x plant part combination was subjected to single factor analysis for strain differences using SAS PROC GLM. Contaminated, missing, or 0 population samples

were not included in the analysis. Within each of these groupings, strain means were compared using Duncan's Multiple Range at alpha = .05.

RESULTS AND DISCUSSION

Log10-transformed population data for each of the strains for each sampling date x plant part combination are compared in Table 14. Basal shoot and basal internode population dynamics are graphically presented in Figure 18.

Strain populations were compared in 8 sampling date x plant part combinations. There was relatively little difference between strains in any of the comparisons. In 5 instances out of 8, there were no significant differences in populations between any of the strains. The only evident trend was that in 7 of the 8 comparisons, populations of the wild-type control (MDE1) were lower than all other strains. Only in 3 of these instances, however, were these populations significantly different from any of the other strains.

CONCLUSIONS

There was relatively little variation between populations of the three strains tested in this experiment.

Table 14

Populations of new construction MDR1.586 and control
strains in various plant parts of greenhouse grown corn,
ranked in descending order.

a.   2 Weeks Post-Inoculation

Basal Shoot		Highest Leaf	
Strain	CFU/g[1]	Strain	CFU/g
MDR1.586	7.50 a	MDR1.586	7.00 a
MDE1	7.12 a	MDE1	6.82 a
MDR1.3	nd[2]	MDR1.3	nd
c.v.[3] = 8.1%		c.v. = 5.4%	

b.   6 Weeks Post-Inoculation

Basal Internode		Sub-ear Internode		Ear Leaf	
Strain	CFU/g[1]	Strain	CFU/g[1]	Strain	CFU/g[1]
MDR1.3	8.57 a	MDR1.3	8.40 a	MDR1.3	7.13 a
MDR1.586	8.15 ab	MDR1.586	8.08 ab	MDR1.586	7.11 a
MDE1	8.00 b	MDE1	7.80 b	MDE1	6.63 a
c.v. = 4.2%		c.v. = 4.9%		c.v. = 7.2%	

c.   10 Weeks Post-Inoculation

Basal Internode		Sub-ear Internode		Ear Leaf	
Strain	CFU/g[1]	Strain	CFU/g[1]	Strain	CFU/g[1]
MDR1.3	9.02 a	MDR1.586	9.21 a	MDR1.3	9.57 a
MDR1.586	8.99 a	MDR1.3	9.06 a	MDR1.586	9.53 a
MDE1	8.82 a	MDE1	9.04 a	MDE1	8.99 b
c.v. = 2.4%		c.v. = 4.2%		c.v. = 4.0%	

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[1]   Mean $\log_{10}$ CFU/g. Means followed by same letter are not
significantly different by Duncan's Multiple Range Test
(alpha = .05).

[2]   Not done.

[3]   Coefficient of variation.

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EXAMPLE 10

THE PRODUCTION OF BT ANTIGEN BY A CXC/BT CONSTRUCT

This study assessed the amount and molecular weight
distribution of Bt endotoxin made by a new Cxc/Bt
construction. The test microbe consists of a Maryland strain
of Clavibacter xyli subsp. cynodontis (Cxc) that has been
genetically engineered to produce the delta endotoxin of
Bacillus thuringiensis subsp. kurstaki ("Bt") and designated
as MDR1.586.

MATERIALS AND METHODS

Test Microbe

The test microbe used in this study, MDR1.586, is
described above in Example 6.

Test System

The basis for quantitation of total Bt antigen in strain
MDR1.586 was immunovisualization of Bt protoxin on Western
Blots (1) of SDS-Polyacrylamide gels. This technique allowed
the determination of a standard curve relating amounts of
pure solubilized Bt crystal protein with the intensities of
color produced with an enzyme-linked second antibody.
Quantitative densitometry was employed to determine values of
color intensity. The test cell extract samples were
solubilized and treated identically to the Bt standards, and
the amount of Bt antigen determined by reference to the
standard curve.

Bt antigen produced by Cxc reflects degradation products
of the Bt molecule as well as the expected 130 kd protein
coded by the native Bt gene. The distribution of molecular
weight species in strain MDR1.586 was also determined by
scanning densitometry.

ANALYTICAL METHODS

Preparation of Cxc Cell Extracts

A culture of strain MDR1.586 was grown in 50 ml S27
medium to mid-log (A660 = 0.5) at 30°C in shaken, baffled
flasks. The culture was chilled on ice, and cells were
harvested by centrifugation. The cells were washed with a
solution containing TRIS-HCl (25mM, pH 6.8), leupeptin (0.5

sg/ml), phenylmethyl sulfonyl fluoride (34 sg/ml) and
disodium EDTA (1 mM) at 0°C. They were then resuspended in 1
ml of the same solution. The cells were disrupted by 3 min
ballistic disintegration in a "Bead-Beater" apparatus
(BioSpec Products, Bartlesville, OK). The resulting lysates
were centrifuged lightly to sediment the glass beads, and the
extracts stored at -20°C.

Quantitation of Protein

Crude cell protein and Bt crystal protein were
determined by the Bradford method (Bio-Rad) with bovine serum
albumin as reference. Both cell extract samples and Bt
crystals were solubilized with 5% NH4OH and 12.5 mM
mercaptoethanol prior to assay.

Preparation of the Bt Crystal Protein Standard

Bt crystals were prepared from B. thuringiensis subsp.
kurstaki HD73 and purified on Renograffin gradients.

Immunochemical Detection of Bt antigen

For quantitation and molecular weight distribution
analysis of Bt antigen, samples of the cell extracts were
solubilized in 1% SDS sample buffer and applied to 4-20%
gradient polyacrylamide-SDS gels (Diichi Pure Chemicals,
Tokyo, Japan). After electrophoresis the gels were Western
blotted (1) onto Immobilon paper (Millipore, Inc.) in an LKB
Transblot apparatus according to manufacturer's instructions.
The Immobilon blot was blocked with skim milk 18 hr at 21°C.
The first antibody, a goat anti-Bt preparation, was added to
the paper and incubated 3 hr at 21°C. After 4 washes to
remove the first antibody a second antibody, rabbit anti-goat
IgG conjugated with alkaline phosphatase (KPL laboratories),
was added and incubated 1 hr at 21°C. The second antibody
was removed by washing and the color reaction initiated by
the addition of the colorigenic substrates (KPL
laboratories). After development the filter was washed with
water to halt the reaction. The filters were stored dry
prior to scanning densitometry.

<u>Densitometry</u>

Filters with immunochemically visualized Bt antigen were scanned with an LKB Model 2222-020 Ultroscan. The density output in area (absorbence units x mm2) was used to construct the Bt standard curve and to compute values for the cell extract samples including the relative contribution of each peak to the total antigenic profile.

RESULTS

<u>Quantitation of total Bt antigen produced by strain MDR1.586.</u>

Fig. 19 shows the standard curve of densitometric units as a function of 130 kdalton Bt crystal protein. Table 15 shows the absorbence values obtained for the 130 kdalton Bt protoxin of the cell extracts, and the conversion of these to weights of Bt protoxin. The corresponding sample values for MDR1.586 cell extracts give a value of 1.3% as the amount of Bt protoxin in cell extract protein.

<u>Contribution of different molecular weight forms of Bt antigen to the total expression.</u>

Fig. 20 shows the scan of Bt antigen as a function of mobility of the different bands. Quantitation of peak areas gave the relative proportions of each band to the total. The major band (32% of total antigen) is the 130 kd native Bt protoxin.

CONCLUSIONS

Strain MDR1.586 produces approximately 10-fold more Bt than the previously found with strain MDR1.3 and this is reflected in the increased toxicity to the European Corn Borer in planta (see Example 7).

Table 15

Quantitation of 130 Kdalton Bt protoxin in extracts of strain MDR1.586

Extract Sample	Sample Protein (ng)	Area (130 kd Peak)	Protoxin[a] (ng protein)	Percent Total Protein
1	7690	0.37	107	1.3
2	7690	0.36	107	1.3

[a]   Computed from standard curve, Figure 1.

While the processes and products of the present invention have been described primarily in reference to hybrids of bacteria capable of fixing nitrogen or producing avermectins, it is to be understood that, with appropriate changes in the screening procedure, hybrids capable of producing other agricultural chemicals, such as insecticides, herbicides, antifungal agents, antibacterial agents, antiviral agents, plant growth regulators, and solubilized phosphates can be prepared in accordance with the present invention. Moreover, while the foregoing examples illustrate the inclusion of genes for production of one agricultural chemical in the hybrid, it is envisioned that genes for production of two or more compatible products can be included in hybrids found in accordance with the present invention. Thus, fusion of hybrids formed in accordance with the present invention with other agricultural-chemical-producing microorganisms, initial fusion with microorganisms known to produce more than one agricultural chemical, or initial fusion conducted with more than one agricultural chemical producing microorganism would yield such multi-chemical-producing organisms. Similarly, two or more genes for the production of agricultural chemicals can be cloned into an infecting microorganism, using recombinant DNA techniques. For example, the genes for the delta-endotoxins of Bacillus, thuringiensis var. kurstaki and Bacillus thuringiensis var. tenebrionis could be cloned into an infecting microorganism.

In addition, the scope of applicable plant hosts can be
expanded by combining the genetic material of two or more
infecting microorganisms.

It will be apparent to those skilled in the art that
various modifications and variations can be made in the
processes and products of the present invention. Thus, it is
intended that the present invention cover the modifications
and variations of this invention provided that they come
within the scope of the appended claims and their
equivalents.

WHAT IS CLAIMED IS:

    1.   A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprising:

        (A)   in any convenient order:

            (1) identifying said plant host; and

            (2) identifying an infecting microorganism which infects said plant host;

        (B)   preparing a vector capable of being transferred into and replicating in said infecting microorganism;

        (C)   preparing an expression module capable of directing the production of an agricultural chemical by said infecting microorganism;

        (D)   placing said expression module in said vector to create an expression vector capable of being transferred into and replicating in said infecting microorganism, said expression vector being capable of directing the production of said agricultural chemical by said infecting microorganism;

        (E)   transforming said infecting microorganism with said expression vector to produce hybrid microorganisms;

        (F)   selecting for said hybrid microorganisms;

        (G)   selecting or screening serially from said selected hybrid microorganisms and in any convenient order:

            (1) a subgroup comprising those hybrid microorganisms which manifest the ability to interact with plant tissue in the manner in which said infecting microorganism interacts with plant tissue during the initial phase of infection in said plant host;

            (2) a subgroup comprising those hybrid microorganisms which, upon application to

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said host, do not create manifestations of disease; and

(3)   a subgroup comprising those hybrid microorganisms having the ability to produce said agricultural chemical if not previously selected for; and

(H)   selecting hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host by selecting from the products of the last performed step of steps (G)(1) to (G)(3) those hybrid microorganisms capable of improving the performance of said plant host under conditions wherein said performance would be improved by direct application of said agricultural chemical to said plant host;

wherein said expression module comprises a gene obtained from the group consisting of microorganisms, plants, vertebrates, and invertebrates.

2.   The method of claim 1 wherein said step of preparing an expression module comprises:

preparing a portable DNA sequence containing the structural gene or genes for said agricultural chemical;

cloning said portable DNA sequence into a vector containing transcription and translation control elements for said portable DNA sequence, said control elements being operable in said infecting microorganism, to create an expression module, said expression module being capable of directing said infecting microorganism to produce said agricultural chemical; and

recovering said expression module.

3.   A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host comprising:

(A)   in any convenient order:

(1)   identifying said plant host; and

        (2)   identifying an infecting microorganism
              which infects said plant host;

(B)   preparing an integration vector containing an
integration sequence and capable of
integrating into the genome of said infecting
microorganism;

(C)   preparing an expression module capable of
directing the production of an agricultural
chemical by said infecting microorganism;

(D)   placing said expression module within aid
integration sequence of said integration
vector, thereby producing a modified
integration vector capable of integrating into
the genome of said infecting microorganism and
directing the production of said agricultural
chemical by said infecting microorganism;

(E)   transforming said infecting microorganism with
said modified integration vector to produce
hybrid microorganisms;

(F)   selecting for said hybrid microorganisms;

(G)   selecting serially from said selected hybrid
microorganisms and in any convenient order:

        (1)   a subgroup comprising those hybrid
              microorganisms which manifest the ability
              to interact with plant tissue in the
              manner in which said infecting
              microorganism interacts with plant tissue
              during the initial phase of infection in
              said plant host;

        (2)   a subgroup comprising those hybrid
              microorganisms which, upon application to
              said host, do not create manifestations
              of disease; and

        (3)   a subgroup comprising those hybrid
              microorganisms having the ability to
              produce said agricultural chemical if not
              previously selected for; and

(H)   selecting hybrid agricultural-chemical-
       producing microorganisms capable of entering
       into endosymbiotic relationships with a plant
       host by selecting from the products of the
       last performed step of steps (G)(1) to (G)(3)
       those hybrid microorganisms capable of
       improving the performance of said plant host
       under conditions wherein said performance
       would be improved by direct application of
       said agricultural chemical to said plant host;

wherein said expression module comprises a gene obtained
from the group consisting of microorganisms, plants,
vertebrates, and invertebrates.

4.   The method of 3 wherein said expression module
comprises a portable DNA sequence containing the structural
gene or genes for the production of said agricultural
chemical and transcription and translation control elements,
said control elements being operable in said infecting
microorganism.

5. The method of 4 wherein said expression module
further comprises a DNA sequence that codes for a selectable
trait in said infecting microorganism.

6.   The method of 3 wherein said step of preparing an
expression module comprises:

preparing a vector containing a promoter that is
operable in said infecting microorganism;

placing into said vector a portable DNA sequence
containing the structural gene or genes for the production of
said agricultural chemical and containing transcription and
translation control elements other than a promoter said
control elements being operable in said infecting
microorganism, to produce an expression vector containing an
expression module, said expression module comprising said
portable DNA sequence and said control elements and said
expression module being operable in said infecting
microorganism; and

recovering said expression module from said expression vector.

7. The method of claim 6 comprising the further step of placing into said vector a DNA sequence that codes for a selectable trait in said infecting microorganism, wherein said expression module further comprises said DNA sequence that codes for said selectable trait.

8. The method of claim 7 wherein said DNA sequence that codes for a selectable trait in said infecting microorganism is within said expression module.

9. The method of claim 3 wherein said infecting microorganism is a microorganism selected from the group consisting of bacteria, fungi, and alga.

10. The method of claim 9 wherein the bacteria is of a genus selected from the group consisting of <u>Acetobacter</u>, <u>Agrobacteria</u>, <u>Azospirillum</u>, <u>Clavibacter</u>, <u>Corynebacteria</u>, <u>Erwinia</u>, <u>Herbispirillum</u>, <u>Pseudomonas</u>, <u>Streptomyces</u>, <u>Xanthomonas</u>, and <u>xylem-limited bacteria</u>.

11. The method of claim 10 wherein said <u>Clavibacter</u> is of the species <u>xyli</u>.

12. The method of claim 11 wherein said plant host is of the Gramineae family.

13. The method of claim 12 wherein said plant host is bermuda grass, sugar cane, or sorghum.

14. The method of claim 11 wherein said plant host is corn.

15. The method of claim 11 wherein said bacterium is <u>Clavibacter xyli</u> subsp. <u>cynodontis</u> and said plant host is corn.

16. The method of claim 11 wherein said bacterium is <u>Clavibacter xyli</u> subsp. <u>cynodontis</u> and said plant host is corn.

17. The method of claim 3 wherein said agricultural chemical is a crop protection agent selected from the group consisting of lysozymes, 1,3-glucanases, chitinases, cecropins, attacins, magainins, protease inhibitors, and polyketides.

18.   The method of claim 3 wherein said integration
vector is pCG300.

19.   The method of claim 6 wherein said vector
containing a promoter is pCG6.

20.   A stable hybrid microorganism having the ability to
produce an agricultural chemical and the ability to enter
into nonpathogenic, endosymbiotic relationships with a plant
host, wherein said agricultural chemical is a crop protection
agent selected from the group consisting of lysozymes, 1,3-
glucanases, chitinases, cecropins, attacins, magainins,
protease inhibitors, and polyketides.

21. The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 1.

22.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 3.

23.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 11.

24.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 12.

25. The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 13.

26.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 14.

27.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 15.

28.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 16.

29.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 17.

30.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 18.

31.   The hybrid agricultural-chemical-producing-
microorganism produced by the method of claim 19.

32.   An agricultural seed product comprising crop plant
seed, a biodegradable nutrient carrier material coated on
said seed, and a stable hybrid microorganism having the
ability to produce agricultural chemicals and to enter into

nonpathogenic endosymbiotic relationships with said crop
plant associated with said carrier material wherein said
agricultural chemical is a crop protection agent selected
from the group consisting of lysozymes, 1,3-glucanases,
chitinases, cecropins, attacins, magainins, protease
inhibitors, and polyketides.

33. An agricultural product comprising crop plant seed
infected with a stable hybrid microorganism having the
ability to produce agricultural chemicals and to enter into
nonpathogenic endosymbiotic relationships with said crop
plant, wherein said agricultural chemical is a crop
protection agent selected from the group consisting of
lysozymes, 1,3-glucanases, chitinases, cecropins, attacins,
magainins, protease inhibitors, and polyketides.

34. An agricultural soil drench comprising a mixture of
water and a stable hybrid microorganism having the ability to
produce agricultural chemicals and to enter into
nonpathogenic, endosymbiotic relationships with a plant host,
wherein said agricultural chemical is a crop protection agent
selected from the group consisting of lysozymes, 1,3-
glucanases, chitinases, cecropins, attacins, magainins,
protease inhibitors, and polyketides.

35. An agricultural product comprising a stable hybrid
microorganism having the ability to produce agricultural
chemicals and the ability to enter into nonpathogenic,
endosymbiotic relationships with a plant host in a
horticulturally acceptable carrier suitable for injection
into said plant host, wherein said agricultural chemical is a
crop protection agent selected from the group consisting of
lysozymes, 1,3-glucanases, chitinases, cecropins, attacins,
magainins, protease inhibitors, and polyketides.

36. A method of producing hybrid agricultural-chemical-
producing microorganisms capable of entering into
endosymbiotic relationships with a plant host according to
claim 5, 7, or 8, wherein said selectable trait of the
infecting microorganism is selected from a group consisting
of antibiotic resistance, need for nutritional

supplementation, resistance to toxins, or combinations thereof.

37. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein the ability to interact with plant tissue in the manner in which said infecting microorganism interacts with plant tissue during the initial phase of infection in said plant host is an ability selected from the group consisting of the ability to bind to plant cells, the ability to spread through the vascular system of said plant host, and the ability to live in the roots of the plant host without creating symptoms of disease.

38. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a monocotyledonous plant.

39. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a temperate cereal crop plant selected from the group consisting of wheat, triticale, barley, rye or oats.

40. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a grass selected from the group consisting of brome grass, bluegrass, tall fescue grass and bermuda grass.

41. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a tropical grass selected from the group consisting of sugar cane, corn, millet or sorghum.

42. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is rice.

43. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 1 or 3, wherein said plant host is a dicotyledonous plant.

44. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a solanaceous plant selected from the group consisting of potatoes, tomatoes, tobacco, eggplant or pepper.

45. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a brassicaceous plant selected from the group consisting of cauliflower, broccoli, cabbage, kale, or kohlrabi.

46. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a vegetable selected from the group consisting of carrot or parsley.

47. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is an agriculturally grown plant selected from the group consisting of sugar beets, cotton, fruit trees, berry plants or grapes.

48. A method of producing hybrid agricultural-chemical-producing microorganisms capable of entering into endosymbiotic relationships with a plant host according to claim 43, wherein said plant host is a tree species selected from the group consisting of pine, spruce, fir or aspen.

49.   A method of producing hybrid agricultural-chemical-
producing microorganisms capable of entering into
endosymbiotic relationships with a plant host according to
claim 1 or 3, wherein said plant host is a legume selected
from the group consisting of soybeans, alfalfa, clover, field
beans, mung beans, peas and other pulses.

(Figure 1)

(Figure 2)

(Figure 3)

(Figure 4)

(Figure 5)

☒ = TBIA promoter
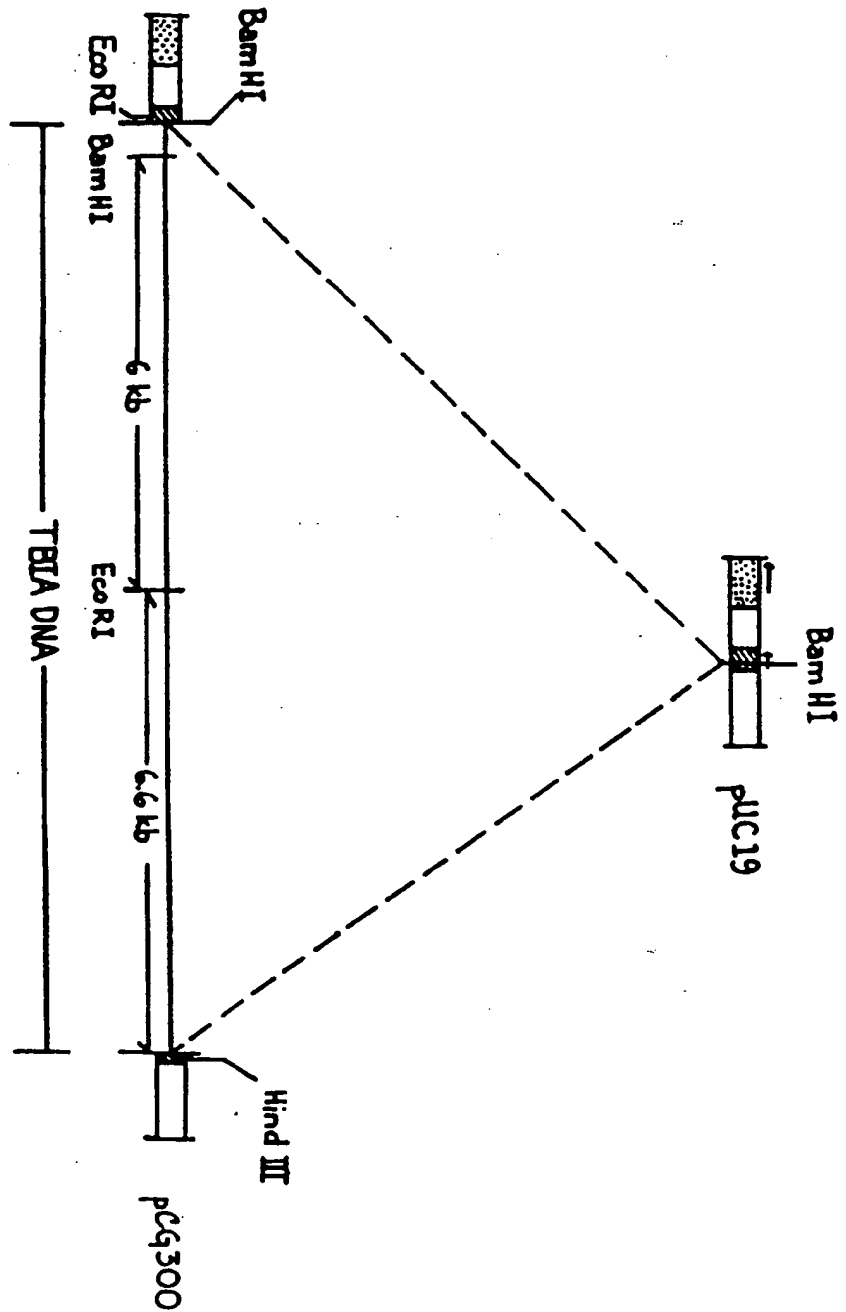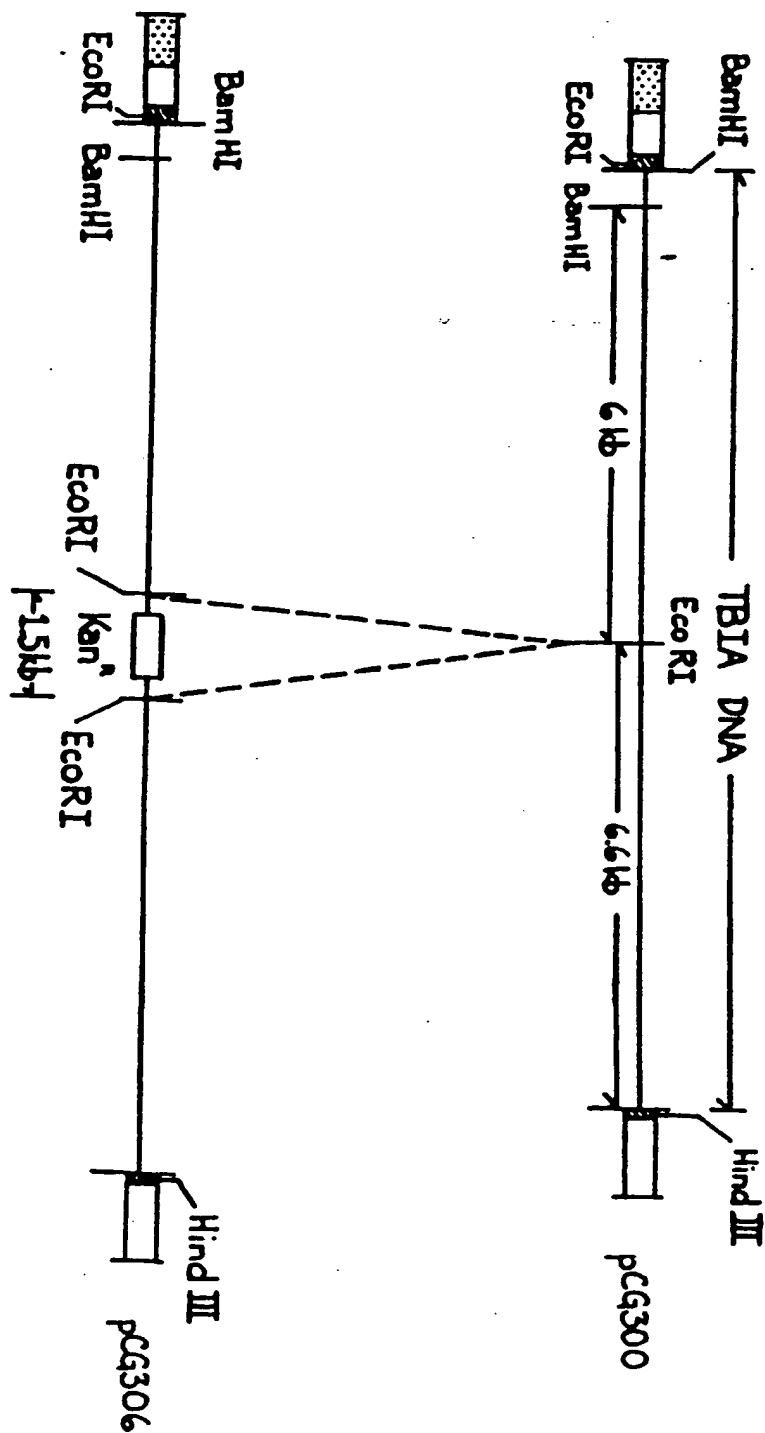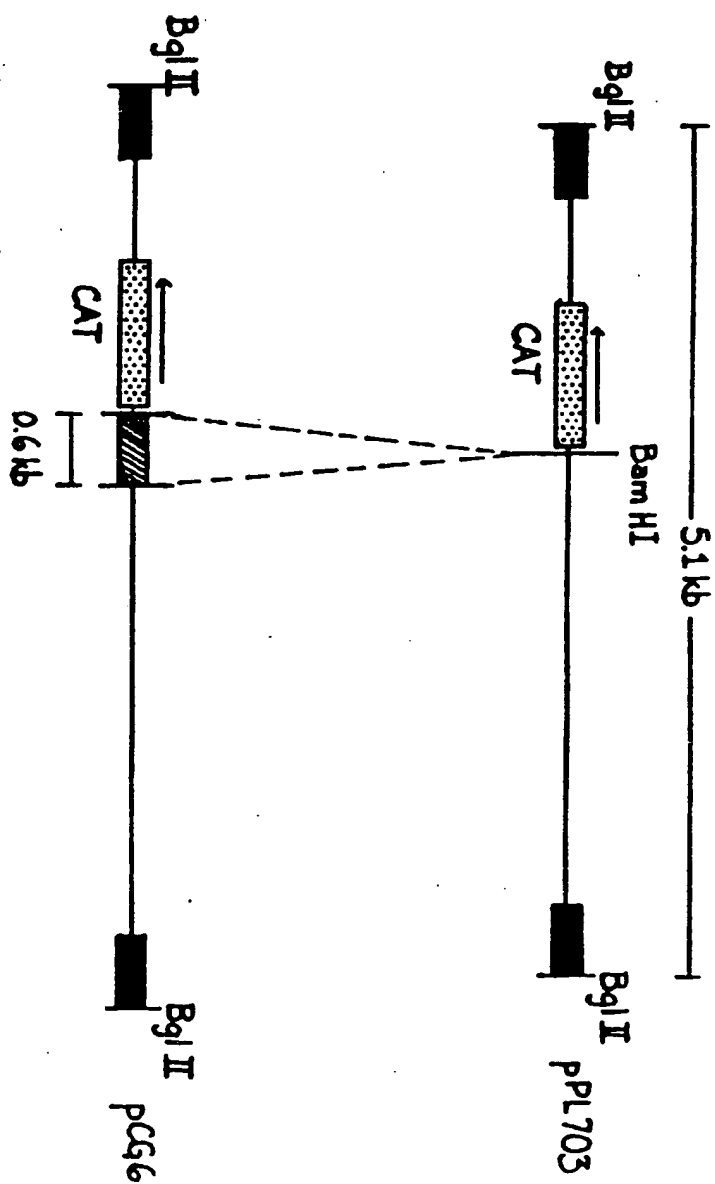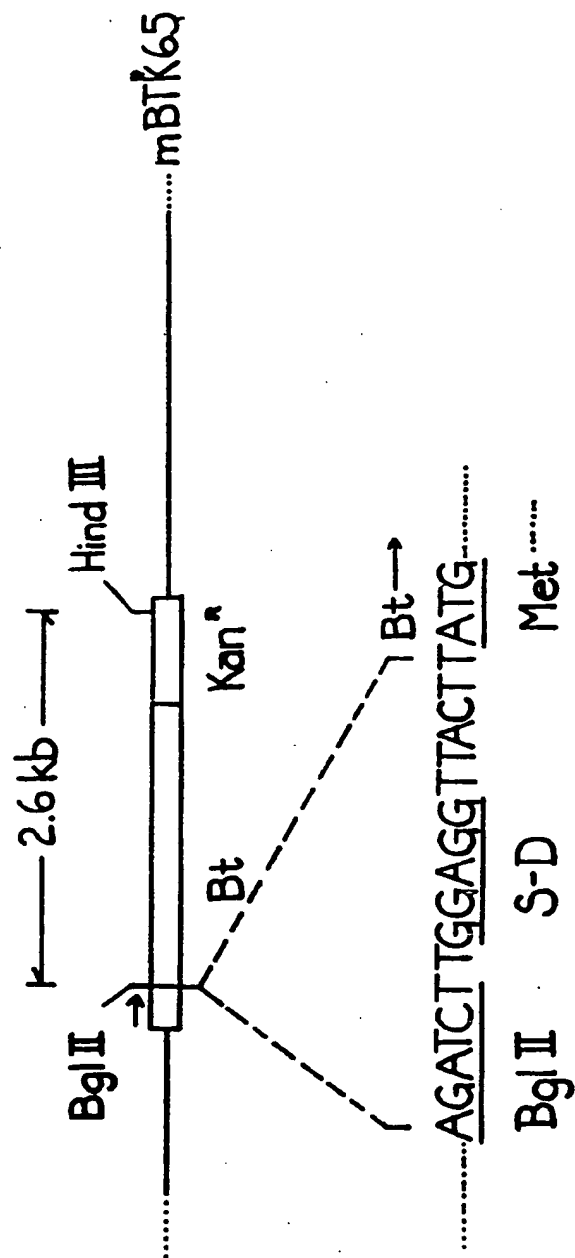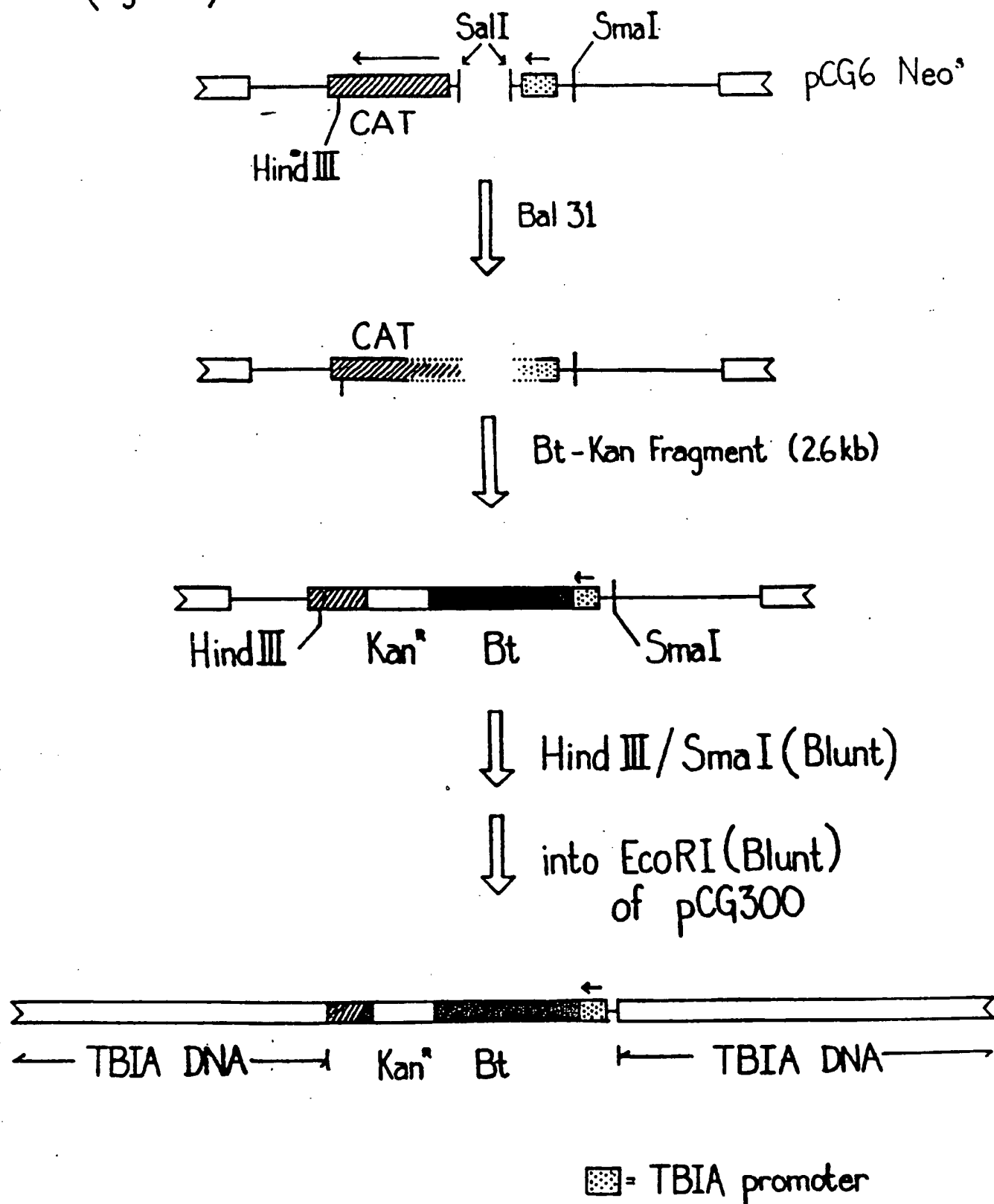
Bt (HD-73)

(4300 bps)
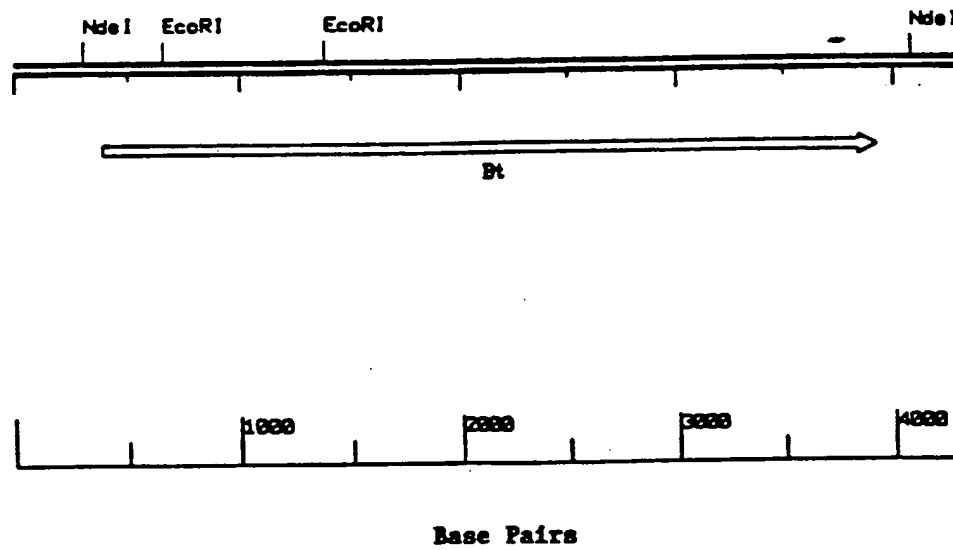
Figure 6

Sequence of 4.993 HincII Fragment of Tn916.

```
   1 CTGCAGGTCAACATACTTCTTGCCATTATACAGGTCCAATCGGGCGGTACTCGGAAGAT   50
  51 GTTATGCAGTCCTCGGAATCCCTCGGTCTTCCACCTAATTCATTGAGTACAGAAGAATCC  120
 121 ATTAAGCAAGGTGTGAAGTATTTCAGTGAATTATTAGCCAGTAGCGAAAGGCTCAGTGTA  180
 181 GATTTAGAATCGGTTATCCAGTCCTACAATTATGGTGGTGGTTTCTTAGGGTATGTGGCT  240
 241 AATCGTGGAAATAAATATACCTTTGAACTGGCTCAAAGTTTCTCAAAAGAGTATTCAGGT  300
 301 GGCGAAAAAGTGTCTTACCCCAATCCCATAGCCATACCTATCAATGGGGGCTGGCGATAC  360
 361 AACTATGGCAATATGTTTTATGTGCAACTGGTAACGCAGTATCTTGTCACAACAGAGTTT  420
 421 GATGATGATACGGTACAAGCCATCATGGACGAAGCACTGAAATATGAGGGCTGGCGATAC  480
 481 GTTTAGGGTGGAGCTTCCCCGACTACTTCTTTTGATTGTAGCGGACTGACACAATGGACG  540
 541 TATGGAAAAGCTGGAATTAACTTACCACGAACCGCACAACAGCAATGATGTGACCCAG  600
 601 CATATCCCACTATCGGAAGCACAAGCTGGCGATTTGGTTTTCTTTCATTCTACCTATAAC  660
 661 GCTGGCTCTTATATTACTCATGTTGGGGATATACCTTGGCAATAACCGTATGTTTCATGCA  720
 721 GGCGACCCAATCGGTTATGCCGACTTAACAAGCCCCTACTGGCAACAGCATTTAGTGGGA  780
 781 GCAGGACGAATCAAACAGTTAGAAAGGGAAGATTTAGTGATGAAATTTAGAAAAAATCAGA  840
 841 ATAAAGAAAAACAGATATCCAAAGGAAAAGAAACCTCGTGTCTATAAGGTCAATCCTCATA  900
 901 AAAAGGTTGTGATTGCCTTGTGGGTACTTTTAGGGCTTAGTTTCAGCTTTCGATATTCA  960
 961 AGCACTTTACAGCTATAGATACTCATACTATTCACGAAACAACTATCATAGAAAAGGAAT 1020
1021 ACGTTGATACTCATCATGTAGAAAATTTTGTAGAGAACTTTGCGAAAGTCTACTATTCAT 1080
1081 GGGAGCAATCCGATAAGTCCATTGATAATCGAATGGAAAGTCTAAAAGGCTATCTGACAG 1140
1141 ATGAACTTCAAGCTCTCAATGTTGATACAGTACGCAAAGATATTCCTGTATCGTCTTCTG 1200
1201 TAAGAGGGATTTCAGATATGGACGGTAGAGCCAACTGGCGACAATGAGTTTAATGTAACCT 1260
1261 ACAGTGTAGACCAGCTCATTACAGAGGGAGAAAATACAAAGACCGTCCACTCTGCTTATA 1320
1321 TAGTGAGTGTCTATGTAGATGGTTCTGGAAATATGGTACTGGTTAAGAATCCGACCATTA 1380
1381 CCAACATACCTAAGAAATCAAGTTATAAACCAAAAGCCATTGAAAGTGAGGGGACGGTTG 1440
1441 ATTCCATTACAACCAATGAAATCAATGAGTTTTTAACGACGTTCTTCAAGCTCTATCCTA 1500
1501 CAGCGACAGCCAGTGAACTTTCCTACTATGTGAATGACGGGATATTAAAACCAATCGGAA 1560
1561 AAGAGTACATCTTTCAAGAACTGGTAAGGATAATCCTATTCACAATCGTAAGGATAATCAAGTCA 1620
1621 CGGTATCGCTGACAGTGGAGTATATCGACCAGCAGACCAAAGCAACGCAGGTATCTCAAT 1680
1681 TTGATTTGGTACTTGAAAAGAACGGGAGTAATTGGAAGATTATAGAATAACAAATATTGG 1740
1741 TACATTATTACAGCTATTTTGTAATCACGTACTCTCTTTGATAAAAAATTGGAGATTCCT 1800
1801 TTACAAATATGCTCTTACGTGCTATTATTTAAGTATCTATTTAAAAGGAGTTAATAAATA 1860
1861 TCCGGCAAGGTATTATTAAATAAACTGTCAATTGATAGCGGGAACAAATAATTGGATGT 1920
1921 CCTTTTTTAGGAGGGCTTAGTTTTTTGTACCCAGTTTAAGAATACCTTTATCATGTGATT 1980
1981 CTAAAGTATCCGGAGAATATCTGTATGCTTTGTATGCCTATGGTTATGCATAAAAATCCC 2040
2041 AGTGATAAGAGTATTTATCACTGGGATTTTTATGCCCTTTTGGGCTTTTGAATGGAGGAA 2100
```

ORF A₃   ORF A₄   TGT M₆   IR₁

```
2101 AATCACGTGAAAATTATTAATATTGGAGTTTTAGCTCATGTTGATGCAGGAAAAACTACC 2160
2161 TTAACAGAAAGCTTATTATATAAACAGTGGAGCGATTACAGAATTAGGAAGCGTGGACAAA 2220
2221 GGTACAACGAGGACGGATAATACGCTTTTAGAACGTCAGAGAGGAATTACAATTCAGACA 2280
2281 GGAATAACCTCTTTTCAGTGGGAAAATACGAAGGTGAACATCATAGACACGCCAGGACAT 2340
2341 ATGGATTTCTTAGCAGAAGTATATCGTTCATTATCAGTTTTAGATGGGGCAATTCTACTG 2400
2401 ATTTCTGCAAAAGATGGCGTACAAGCACAAACTCGTATATTATTTCATGCACTTAGGGAAA 2460
2461 ATGGGGATTCCCACAATCTTTTTTATCAATAAGATTGACCAAAATGGAATTGATTTATCA 2520
2521 ACGGTTTATCAGGATATTAAAGAGAAACTTTCTGCCGAAATTGTAATCAAACAGAAGGTA 2580
2581 GAACTGTATCCTAATGTGTGTGTGACGAACTTTACCGAATCTGAACAATGGGATACGGTA 2640
2641 ATAGAGGGAAACGATGACCTTTTAGAGAAATATATGTCCGGTAAATCATTAGAAGCATTG 2700
2701 GAACTCGAACAAGAGGAAAGCATAAGATTTCAGAATTGTTCTCTGTTCCCTCTTTATCAT 2760
2761 GGAAGTGCAAAAAGTAATATAGGGATTGATAACCTTATAGAAGTTATTACTAATAAATTT 2820
2821 TATTCATCAACACATCGAGGTCCGTCTGAACTTTCGGGAAATGTTTTCAAAATTGAATAT 2880
2881 ACAAAAAAAGACAACGTCTTGCATATATACGCCTTTATAGTGGAGTACTACATTTACGA 2940
2941 GATTCGGTTAGAGTATCAGAAAAAGAAAAAATAAAAGTTACAGAAATGTATACTTCAATA 3000
3001 AATGGTGAATTATGTAAGATTGATAGAGCGTTATTCTGGAGAAATTGTTATTTTGCAAAAT 3060
3061 GAGTTTTTGAAGTTAAATAGTGTTCTTGGAGATACAAAACTATTGCCACAGAGAAAAAG 3120
3121 ATTGAAAATCCGCACCCTCTACTACAAACAACTGTTGAACCGAGTAAACCTGAACAGAGA 3180
3181 GAAATGTTGCTTGATGCCCTTTTGGAAATCTCAGATAGTGATCCGCTTCTACGATATTAC 3240
3241 GTGGATTCTACGACACATGAAATTATACTTTCTTTCTTAGGGAAAGTACAAATGGAAGTG 3300
```

Figure 7

```
      ----.----+----.----+----.----+----.----+----.----+----.----+
3301  ATTAGTGCACTGTTGCAAGAAAAGTATCATGTGGAGATAGAACTAAAAGAGCCTACAGTC  3360
3361  ATTTATATGGAGAGACCGTTAAAAAATGCAGAATATACCATTCACATCGAAGTGCCCGCCA  3420
3421  AATCCTTTCTGGGCTTCCATTGGTTTATCTGTATCACCGCTTCCGTTGGGAAGTGGAATG  3480
3481  CAGTATGAGAGCTCGGTTTCTCTTGGATACTTAAATCAATCATTTCAAAATGCAGTTATG  3540
3541  GAAGGGATACGCTATGGTTGTGAACAAGGATTGTATGGTTGGAATGTGACGGACTGTAAA  3600
      ----.----+----.----+----.----+----.----+----.----+----.----+
3601  ATCTGTTTTAAGTATGGCTTATACTATAGCCCTGTTAGTACCCTAGCAGATTTTCGGATG  3660
3661  CTTGCTCCTATTGTATTGGAACAAGTCTTAAAAAAAGCTGGAACAGAATTGTTAGAGCCA  3720
3721  TATCTTAGTTTTAAAAATTTATGCGCCACAGGAATATCTTTCACGAGCATACAACGATGCT  3780
3781  CCTAAATATTGTGCGAACATCGTAGACACTCAATTGAAAAATAATGAGGTCATTCTTAGT  3840
3841  GGAGAAATCCCTGCTCGGTGTATTCAAGAATATCGTAGTGATTTAACTTTCTTTACAAAT  3900
      ----.----+----.----+----.----+----.----+----.----+----.----+
3901  GGACGTAGTGTTTGTTTAACAGAGTTAAAAGGGTACCATGTTACTACCGGTGAACCTGTT  3960
3961  TGCCAGCCCCGTCGTCCAAATAGTCGGATAGATAAAGTACGATATATGTTCAATAAAATA  4020
4021  ACTTAGTGTATTTTATGTTGTTATATAAATATCGTTTCTTGTTAAATAAGATGAAATATT  4080
4081  TTTTAATAAAGATTTGAATTAAAGTGTAAAGGAGGAGATAGTTATTATAAACTACAAGTG  4110
4141  GATATTGTGTCCTGTATGTGGAAATAAAACACGATTAAAGATAAGGGAAGATACTGAATT  4200
      ----.----+----.----+----.----+----.----+----.----+----.----+
4201  AAAAAAATTCCCCCTCTATTGTCCGAAATGCAGACAAGAAAATTTAATTGAAATAAAGCA  4260
4261  GTTCAAAGTAACTGTGATTACAGAGCCAGACGCAAAGACGCAGAGCCGATAAAATGAGAT  4320
4321  TAATACAATCTCATTTTATCGGCTCTTTCCGTTATGTATGGATTCTTTTAATCGTCTTC  4380
4381  GATGTTTCTTGCTTCGTTGATACCGCTGGCTAAAGATCCATTAAGGATAGTTCTTTGTC  4440
4441  TGTAAAGCTATCCATGTATTTCTCTATCTGTAATCGTCGGGTGCTTTTTTACCAAGTTATT  4500
      ----.----+----.----+----.----+----.----+----.----+----.----+
4501  AGCAGGTAAGAAAAATTCATCAACGGAAACATGAAGTAAGGATACAAGGTCATAAAGAAC  4560
4561  TTGTATGCTGGGGTGTTGCCCGTTATTTTCAATATTAGTTAAGTACCGTGGGTCAATTTC  4620
4621  AATCAATGCTCCCACTTGTTCACGAGTTAAACCTCGTTTCAATCGAGCTTCTTTAATGGC  4680
4681  TAAACCAAAGGCTCTTAAAATCATATTTATCTTCTTTTTACCGTAGTAGACCACCTCTA  4740
4741  TACATTTTATTGTTCCTACTGAATTAAAAACAGGTATAGAAAAACGTGTTATATGGTTTA  4800
      ----.----+----.----+----.----+----.----+----.----+----.----+
4801  TAGGTTTATATTTAATAAAAAGCACTACTAAACGCCAATAAAAAAAACCGTTATATGGTA  4860
4861  GTGCTATTTACGCTGTTAAAATATTGTATATTACTTCCAAATGGCGGTTTGTTGGAGGTC  4920
4921  GACGCGGAAGATGTTATGCAGTCCTCGGAATCCCTCGGTCTTCCACCTAATTCATTGAGT  4980
4981  ACAGAAGAATCCA                                                4993
```

ORF A$_s$ = ATG start of putative gene A (box).
ORF A$_e$ = Termination codon of putative gene A (dashes).
ORF B$_s$ = ATG start of putative gene B (complement) (box).
ORF B$_e$ = Termination codon of putative gene B (complement) (dashes).
IR = Inverted repeat (underline).
TetM$_s$ = ATG start of tetM gene (box).
TetM$_e$ = Termination codon of tetM gene (dashes).

Figure 7
(continued)
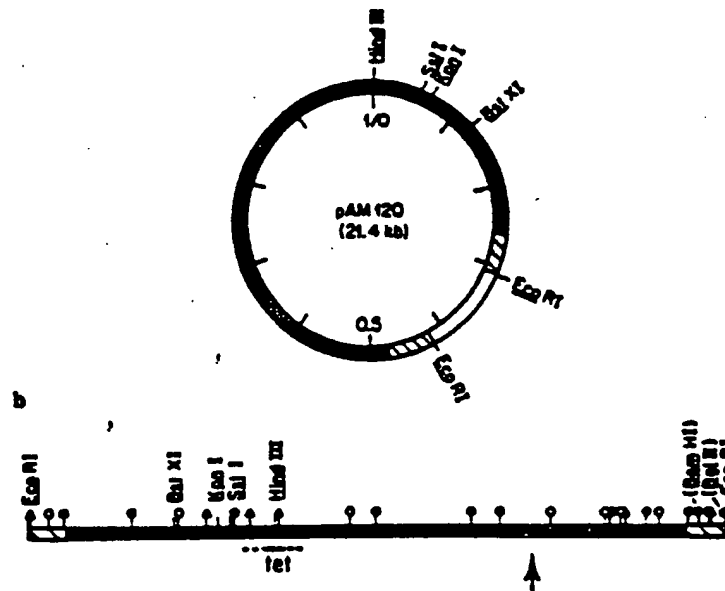
Figure 1 Restriction map of Tn916. (a) pAM120, representing the EcoRI fragment F::Tn916 from pAD1::Tn916 (pAM211) cloned into the E. coli vector pGL101. The dark segment represents Tn916. The striped segments represent the portions of the EcoRI F fragment flanking Tn916. The remainder represents pGL101. (b) Tn916 and its flanking DNA. The restriction sites are indicated or are represented by: filled circle, Sau3A; open circle, HincII; triangle, HpaII. The HindIII site in Tn916 is believed to be within or near tet.

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Figure 8
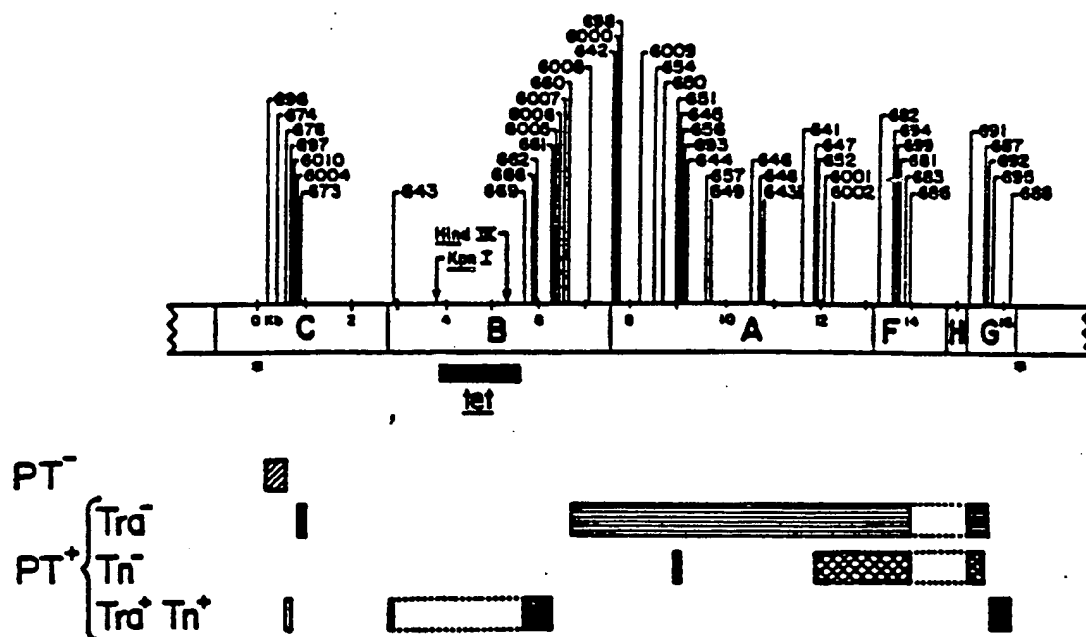
FIG. L. Tn916 restriction map showing insertions of Tn5 and resulting effects on behavior. The asterisks mark the ends of the transposon. The segments marked A (5.5 kb), B (4.3 kb), C (3.6 kb), F (1.6 kb), G (1.1 kb), and H (0.4 kb) correspond to HincII fragments originally identified in pAM120 (14, 22). The numbers correspond to the corresponding pAM derivatives (Table 3) generated. The different behaviors are indicated in Table 3 as follows: PT⁻, unable to transform S. faecalis OG1X protoplasts; Tra⁻, unable to transfer conjugatively; Tn⁻, unable to detect intracellular transposition; and PT⁻ Tra⁺ Tn⁺, behavior similar to wild-type Tn916. The resistance determinant is indicated as tet.

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Figure 9
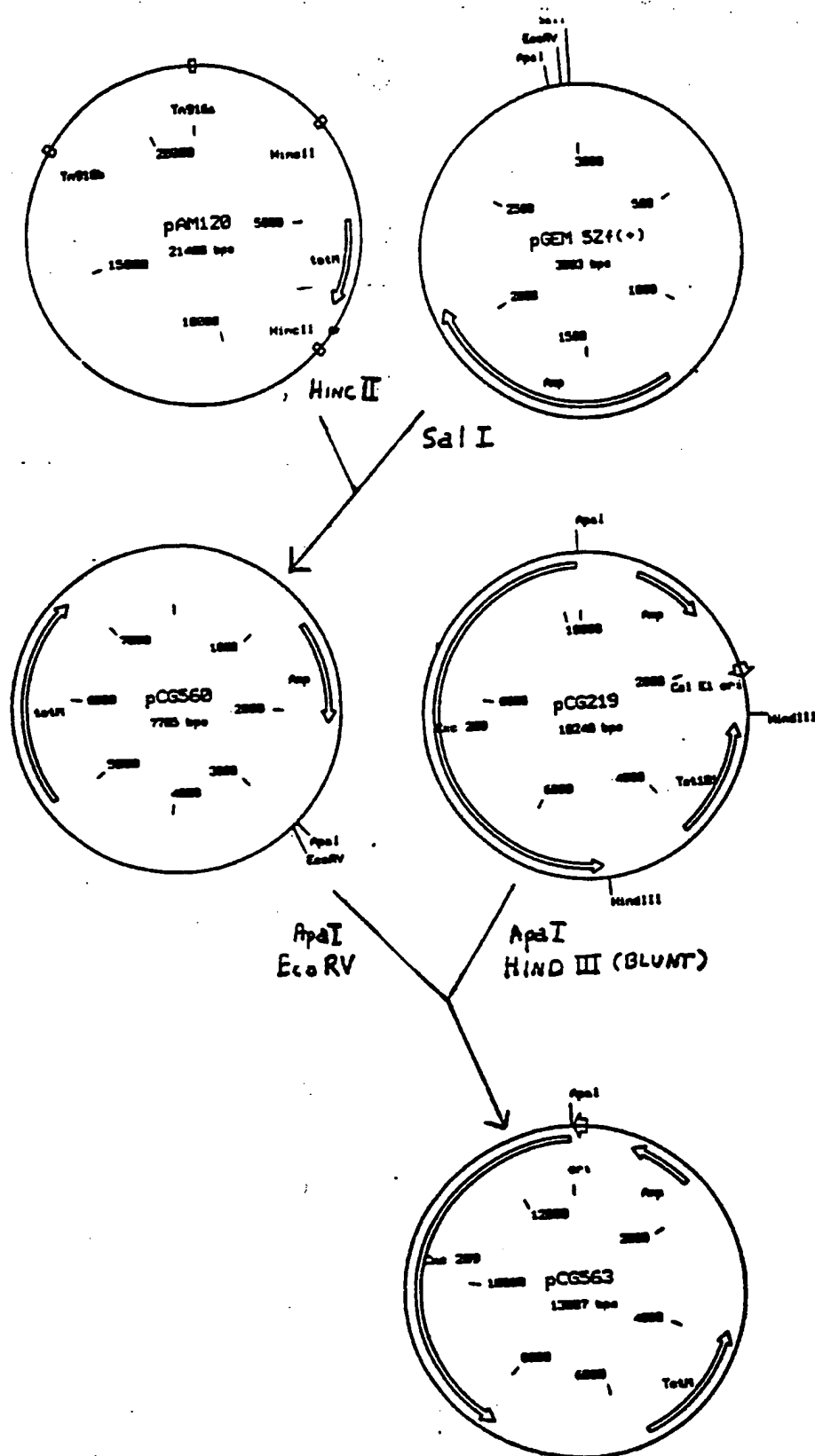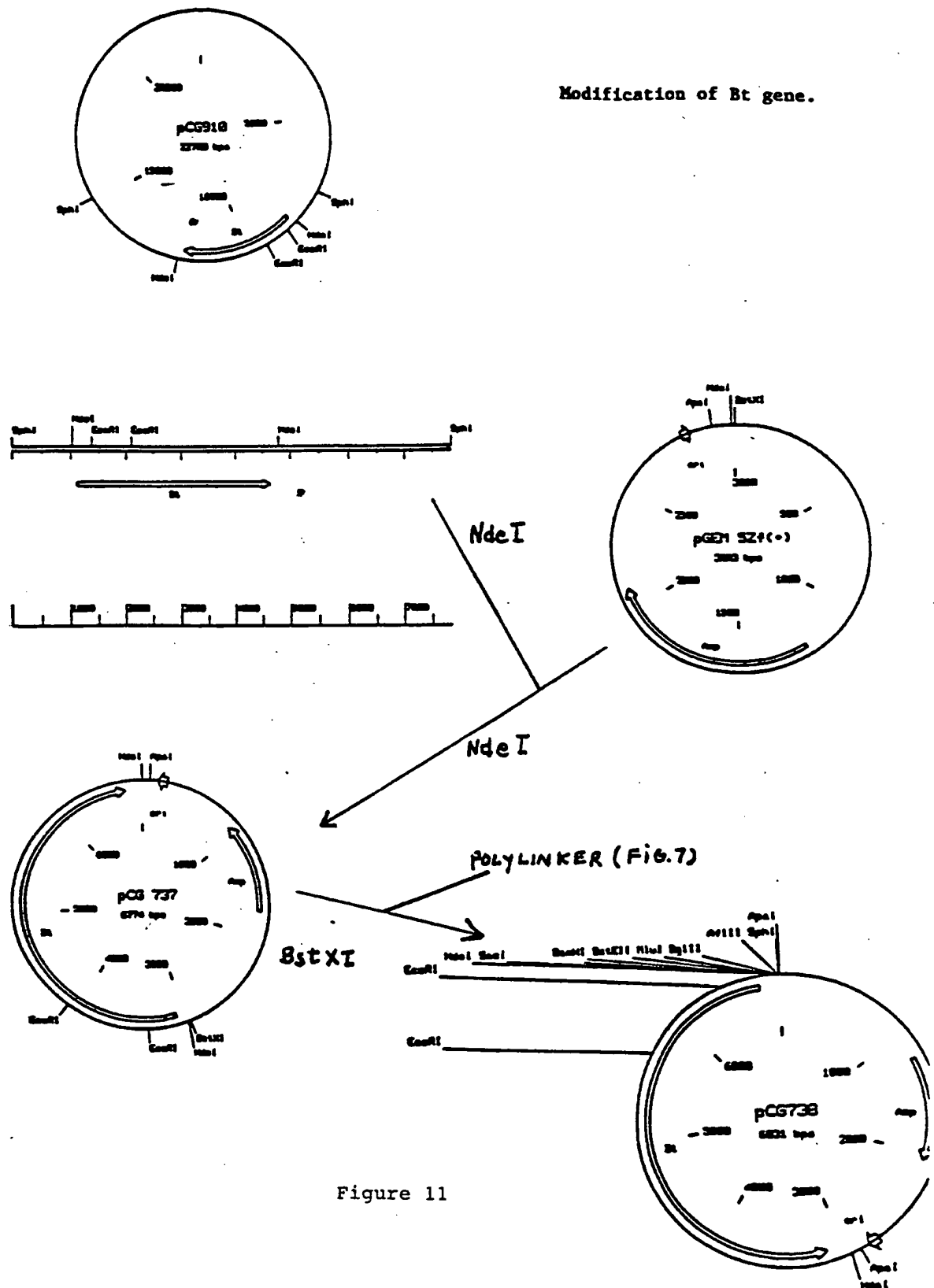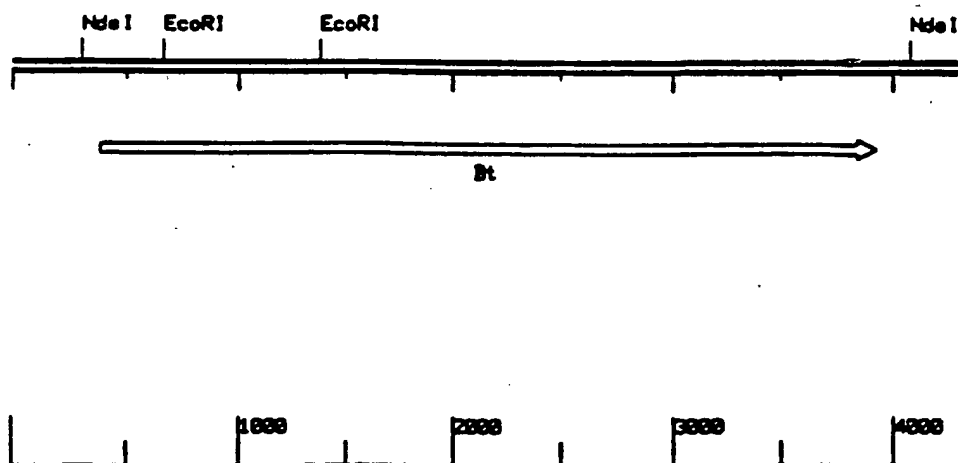
Assembly of pCG563.

Figure 10

Modification of Bt gene.

Figure 11

Figure 12

Final Assembly   pCG741.
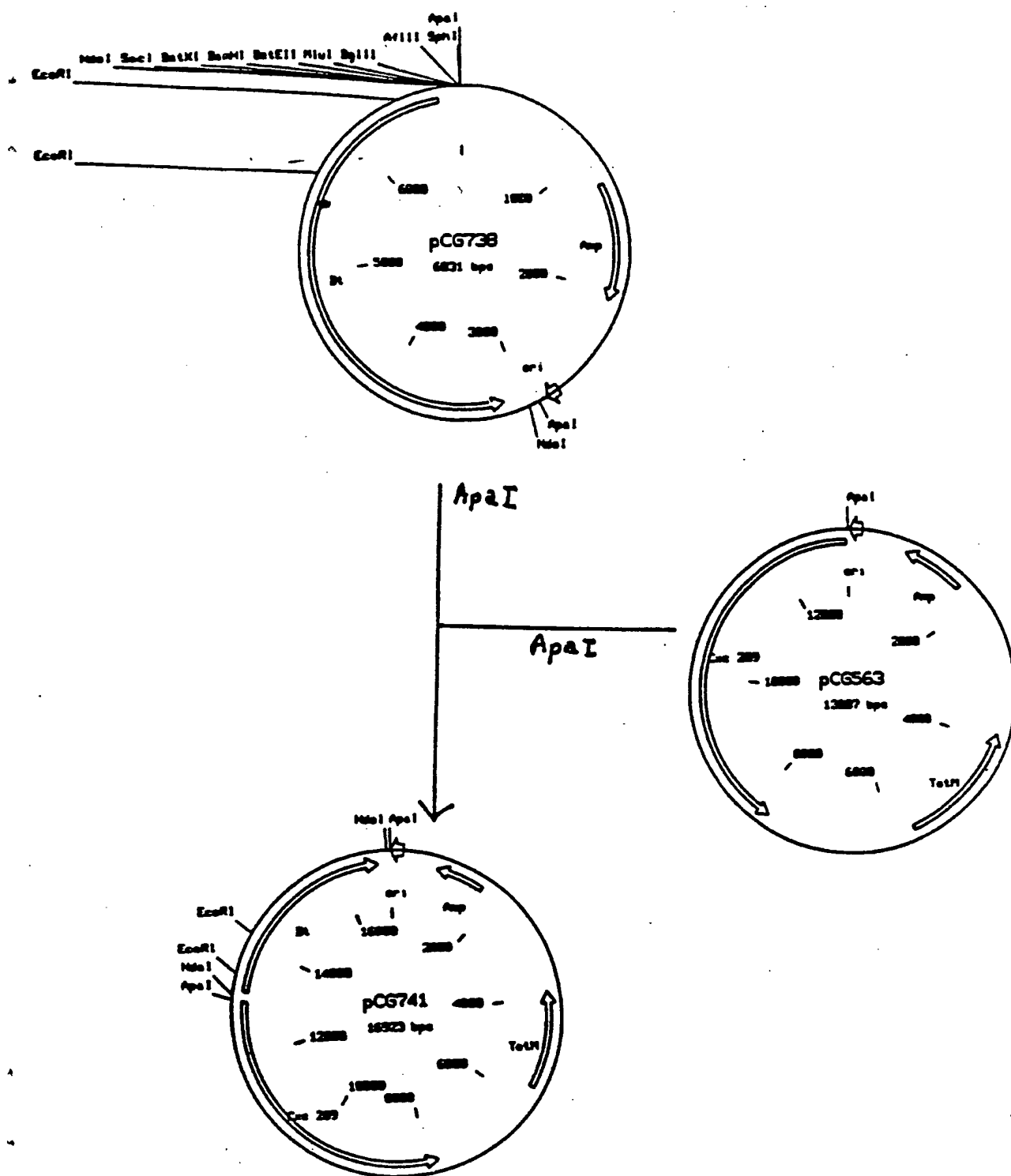
Figure 13

Sou   . of Probes.
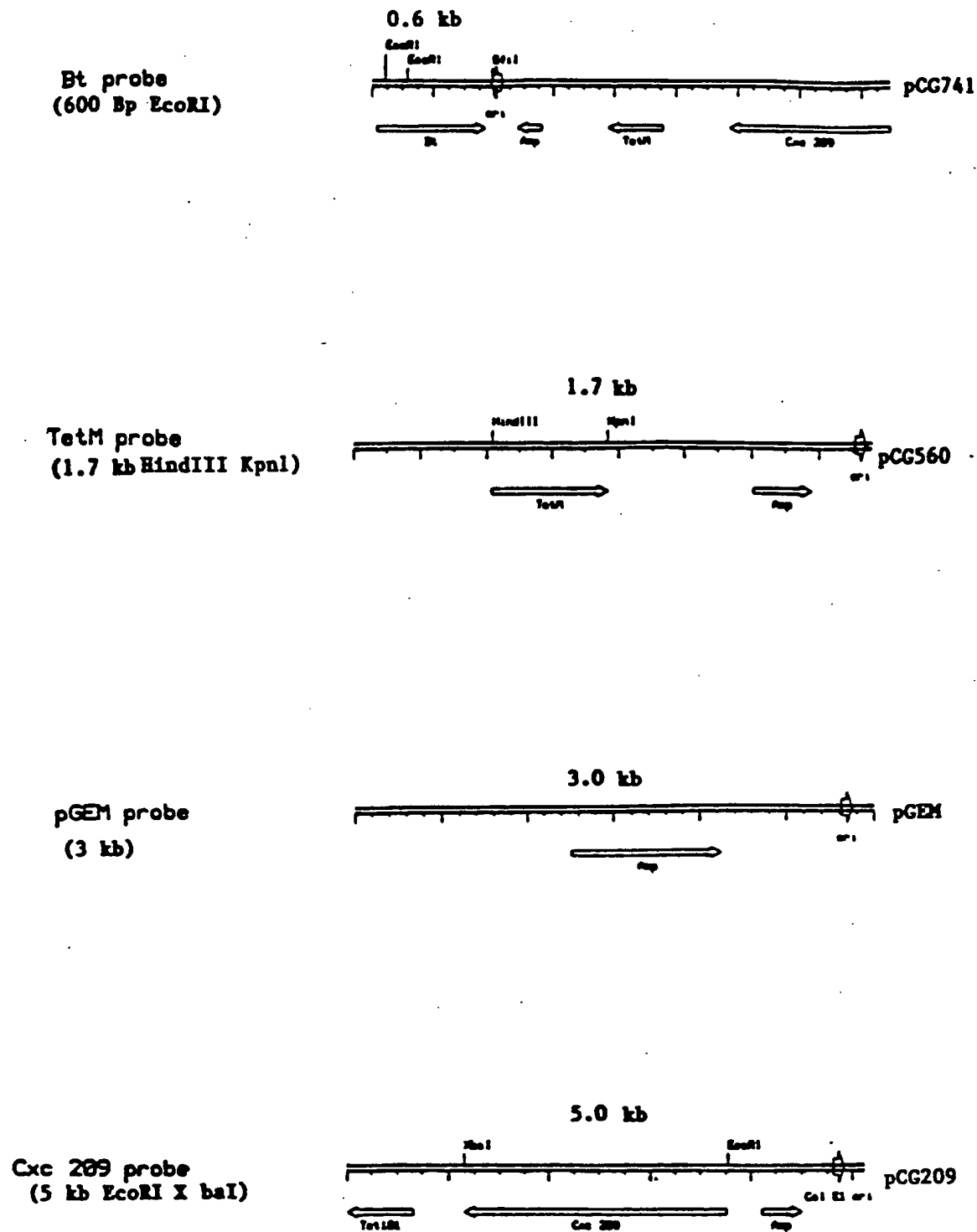
0.6 kb

Bt probe
(600 Bp EcoRI)

pCG741

1.7 kb

TetM probe
(1.7 kb HindIII Kpnl)

pCG560

3.0 kb

pGEM probe
(3 kb)

pGEM

5.0 kb

Cxc 209 probe
(5 kb EcoRI X baI)

pCG209

Figure 14

Restriction enzyme sites used for southern analysis of
pCG741 in MDR1.586.
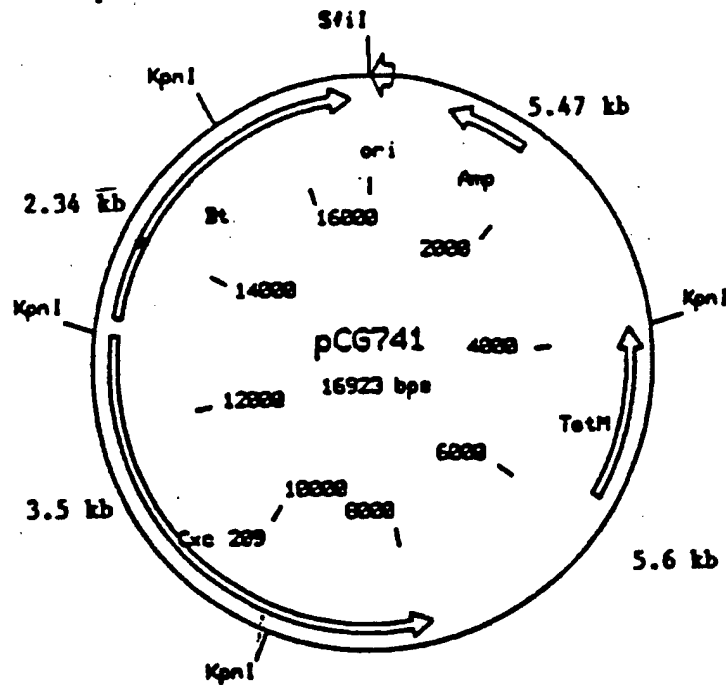
Figure 15
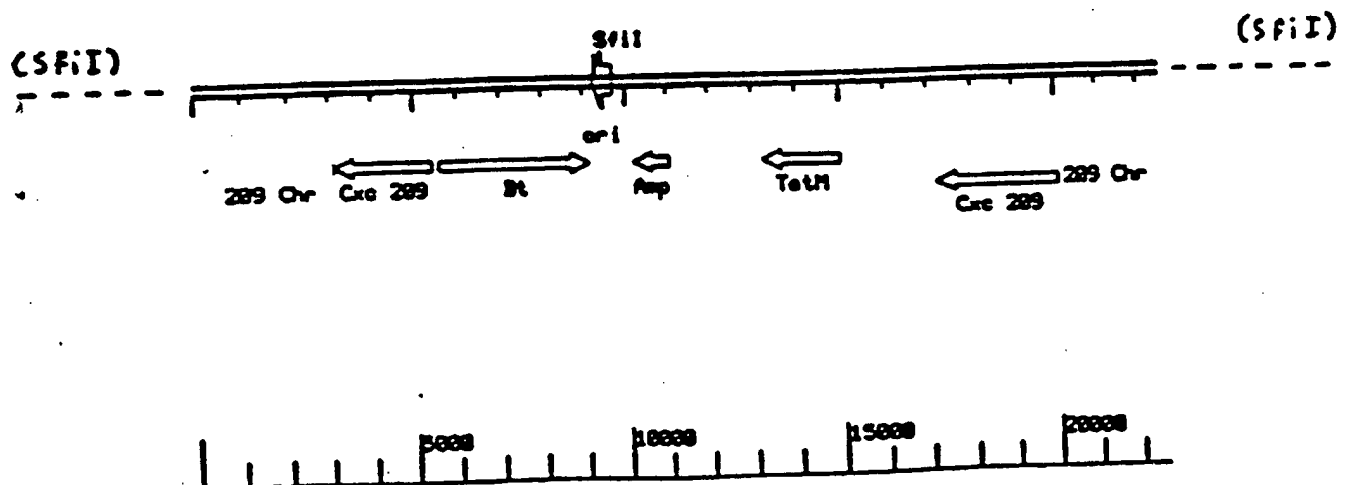
SfiI Digest of DNA Samples: Southern Analysis
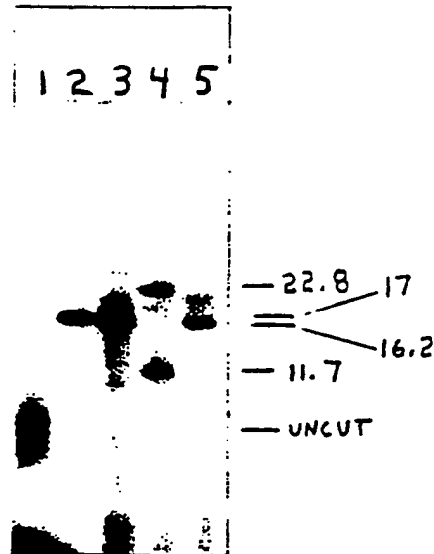
Lane 1 is 5 ng uncut pCG741 pure plasmid; Lane 2 is 5 ng pCG741 cut with SfiI; lane 3 is a mix of 5 ng pCG741 and 2 μg MDE1 chromosomal DNA cut together with SfiI; lane 4 contains 2 μg MDR1.586 chromosomal DNA cut with SfiI; lane 5 contains 2 μg MDE1 chromosomal DNA cut with SfiI. The probe is Cxc 209 DNA (Fig. 9). The resolving gel is 0.4% Agarose/TAE. Molecular weights of fragments are shown on right of figure, computed by reference to ethidium bromide stained markers.

Figure 16

KpnI Digests of DNA Samples: Southern Analysis.
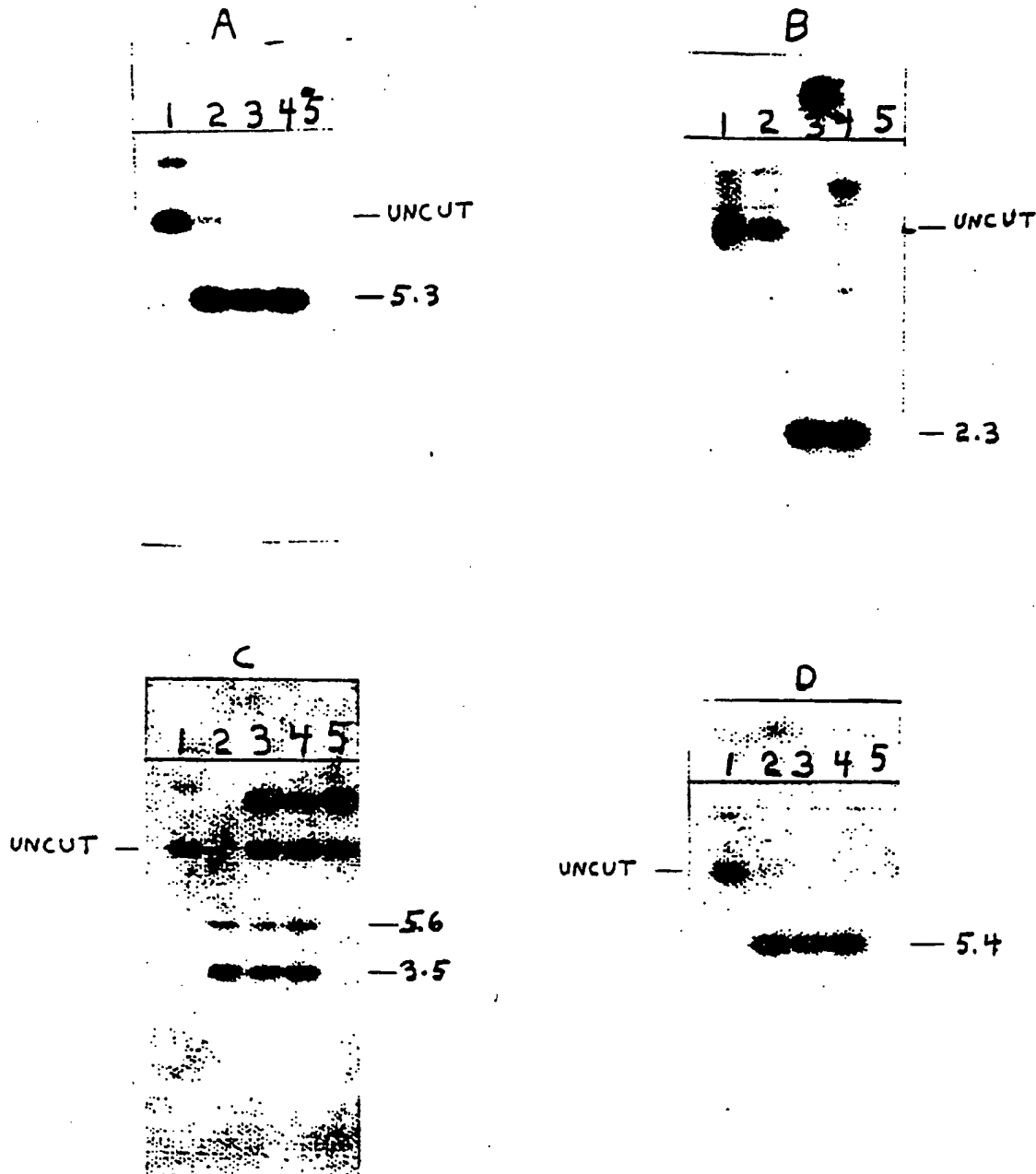
Figure 17

Lane 1 is 5 ng uncut pCG741; lane 2 is 5 ng pCG741 cut with KpnI; lane 3 is a mixture of 5 ng pCG741 and 2 µg MDE1 DNA cut with KpnI; lane 4 is 2 µg MDR1.586 cut with KpnI; lane 5 is 2 µg MDE1 cut with KpnI.

Probes (Fig. 9) are: (A) = pGEM; (B) = Bt; (C) = Cxc209; (D) = tetM. Resolving gel is 1% agarose/TAE. Molecular weights of fragments are given in kb on right of figure. Lane 2 (b) did not receive enough KpnI to cut.

Population dynamics of new construction MDR1.586 and control strains
in the basal shoot (2 weeks) and the basal internode (6 weeks
and 10 weeks) of greenhouse grown corn.
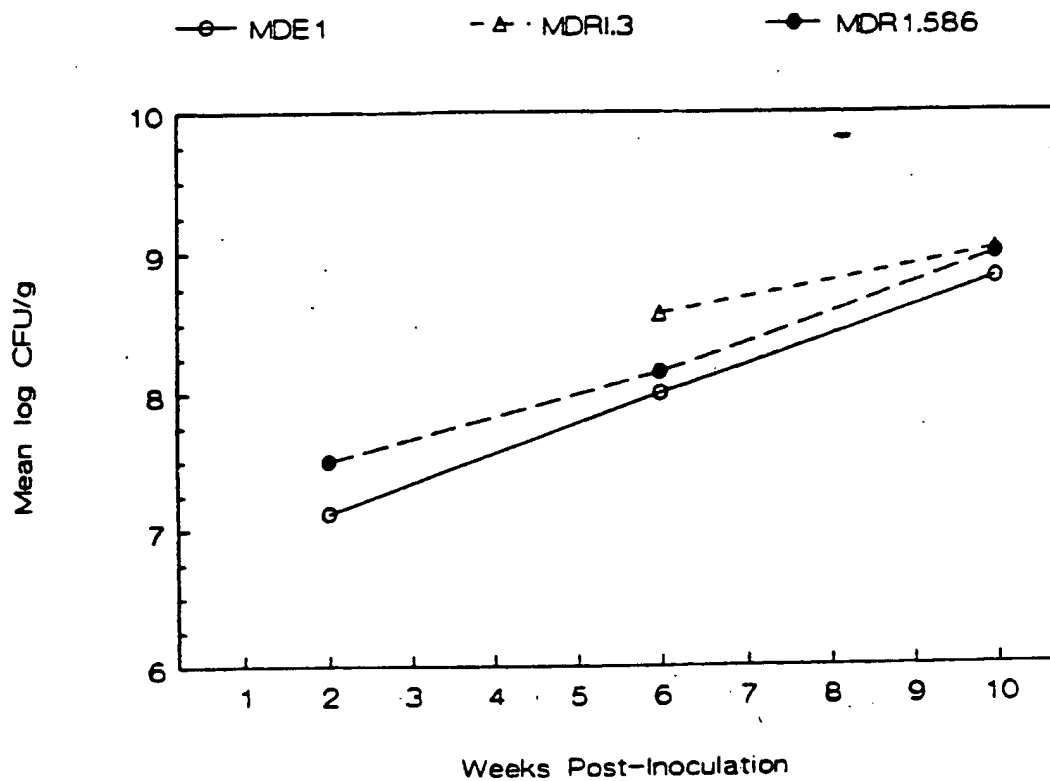
Figure 18
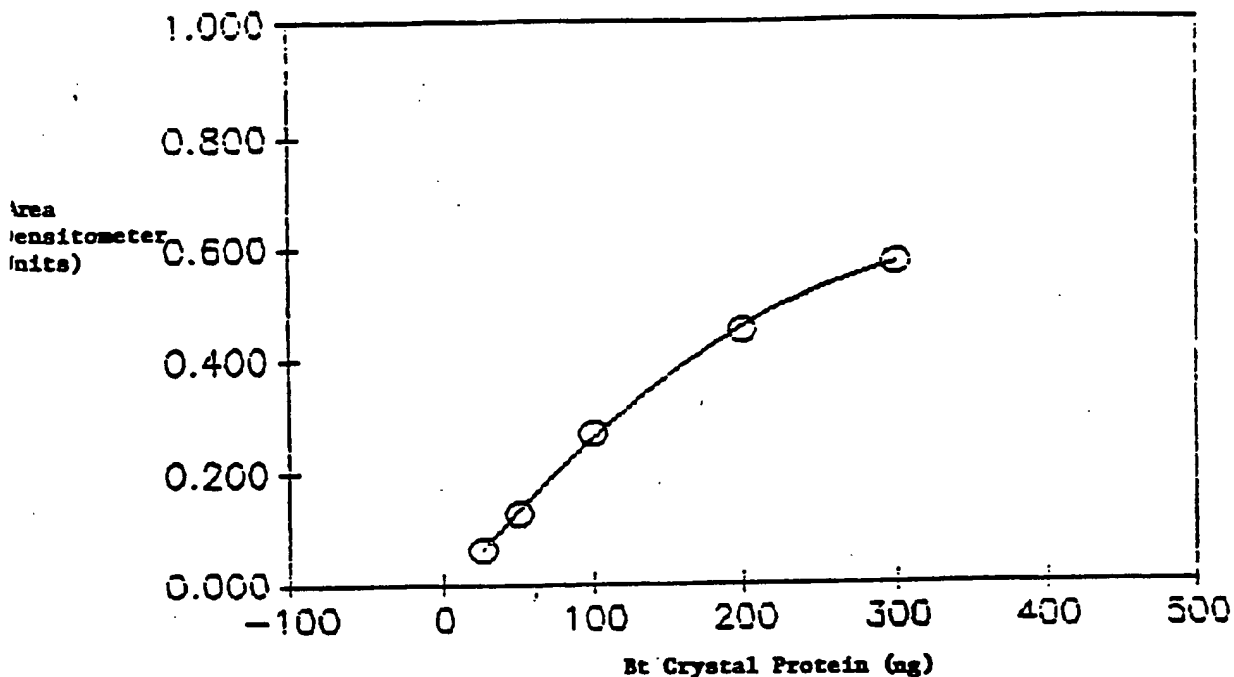
Standard curve generated by scanning densitometry of
immunovisualized Western blot. The standards are
the indicated quantities of Bt crystal protein
solubilized in SDS sample buffer and resolved in a
4-20% SDS polyacrylamide gel. The protein was
electrophoretically blotted onto Immobilon paper and
the 130 kd protoxin visualized by an anti-Bt
(goat):anti-goat (rabbit)(alkaline phosphatase
conjugant) sandwich.

Figure 19
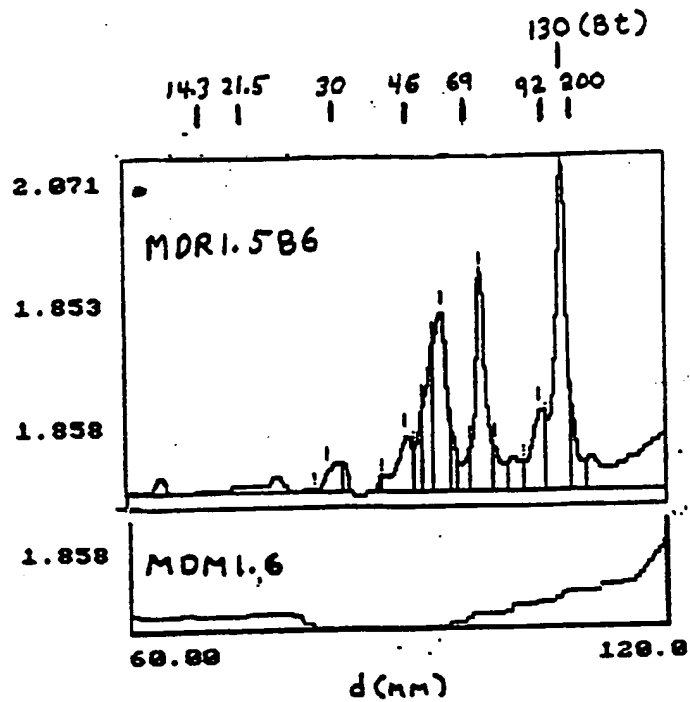
Densitometry of Western Blots.

Densitometric trace of immunovisualized Bt antigen
of strain MDR1.586 (top) and control extract MDM1.6
(bottom). Molecular weight marker positions are
listed by their Kdalton values (top), and the
position of the 130 Kdalton Bt crystal protein is
also shown.

Figure 20